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Chemical biology approaches for the identification of novel p53 regulatory signalling pathways.

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Abstract

p53 is a critical tumour suppressor which acts to repair or remove abnormal cells and thus prevent cancer. Aberrant function of p53 is therefore a critical step in tumourigenesis and p53 is mutated in half of all cancers. Mutation of p53 leads to both a loss of normal wild-type function as well as the gain of oncogenic function. p53 is considered to be a promising therapeutic target and therapeutic strategies for targeting of the p53 pathway include: 1. Activation of wild-type p53 (wtp53) protein function, 2. Refolding of mutant p53 (mtp53) into the wtp53 conformation, 3. Reduction of mtp53 protein levels. In this work a number of small molecule screening assays were used to identify potentially novel regulators of both wtp53 and mtp53. Screening of a protein kinase inhibitor library for small molecules which can stimulate wtp53 activity identified the GSK3 pathway and a CDK pathway as dominant suppressors of wtp53 function. Screening of the library for inhibitors which reduce mtp53 protein levels led to the identification of two IKKβ inhibitors. The work then focused on investigating the effects of one of these compounds, IMD0354, on the mutant p53 pathway; with a specific focus on MDM2 as the most rapidly responding biomarker. IMD0354 is a well characterised inhibitor which has been shown to specifically inhibit IKKβ leading to the repression of the Nf-κB pathway. This study shows that IKKβ inhibition leads to the loss of a number of oncogenic proteins including mtp53, MDM2 and cyclin D. Mass-spectrometry based (ITRAQ) proteomic analysis was then employed to identify potential mediators and/or co-regulated factors in response to IKKβ-inhibition via IMD0354 treatment. This led to the identification of RPS3 as a potential negative regulator of MDM2 protein expression; the reduction in MDM2 protein in response to IMD0354 treatment is shown to be partially dependent on RPS3. Together this data has identified, using small molecule kinase inhibitor libraries: (i) dominant kinase signalling pathways that suppress wt-p53 and (ii) a dominant kinase signalling pathway that sustains expression of mutant p53 and MDM2 in cancer cell lines. This latter data supports further investigation to aid understanding of how the IKK signalling pathway cross-talks to the p53-MDM2 axis.

Declaration

I declare that this thesis has been composed by myself, Emma Rusilowicz, and the work contained is entirely my own unless otherwise acknowledged.
This work has been submitted for the degree of Doctor of Philosophy and has not been submitted for any other degree or professional qualification.

Emma V Rusilowicz

Table of Contents

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Abbreviations:

17-AAG – 17-N-Allylamino-17-demethoxygeldanamycin

ACN - Acetonitrile

AKT – Protein kinase B

ARF-BP1 – ARF binding protein 1

AMP - Adenosine monophosphate

AMPK - AMP-activated protein kinase

AP1 – Activator protein 1

ATM – Ataxia telangiectasia mutated

ATP - Adenosine-5'-triphosphate

BAI1 – Brain specific angiogenesis inhibitor

Bak – Bcl2 agonist/killer 1

Bax – Bcl2 associated X protein

Bcl2 - B-cell CLL/lymphoma 2

β-gal – β- galactosidase

BSA – Bovine serum albumin

Btk - Bruton's tyrosine kinase

β-TrCP1 - Beta-transducin repeat containing E3 ubiquitin protein ligase

cAMP – Cyclic AMP

Cdk – Cyclin dependent kinase

CDKN2A - Cyclin-dependent kinase inhibitor 2A

cDNA – Complementary DNA

CHIP – Carboxy terminus of Hsp70-interacting protein

cIAP2 - Cellular inhibitor of apoptosis 2

CK1 - Casein kinase 1

CMV – Cytomegalovirus

COP-1 - Constitutive photomorphogenesis protein 1

C-REG – C-terminal regulatory domain

CRM1 - Chromosome Region Maintenance 1

DBD – DNA binding domain

DM - Dorsomorphin

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethylsulfoxide

DNA - Deoxyribonucleic acid

DNMT3A – DNA (cytosine-5)-methyltransferase 3A

DTT – Dithiothreitol

ECL – Enhanced chemiluminescent reagent

EDTA - Ethylene diamine tetraacetic acid

EGFR - Epidermal growth factor receptor

EGR-1 - Early growth response protein 1

EGTA – Ethylene glycol tetraacetic acid

ERK - Extracellular signal-regulated kinases

FAK – Focal adhesion kinase

FBS – Foetal bovine serum

FISH – Fluorescent *in situ* hybridisation

Fluorosilane - Tridecafluoro-1,1,2,2-tetrahydrooctyl-dimethylchlorosilane

GADD45 – Growth arrest and DNA-damage-inducible protein 45

GF - GF109203X

GPCR – G-Protein coupled receptor

GRK2 - G protein–coupled receptor kinase 2

GS - Genistein

GSK-3 - Glycogen synthase kinase 3

Hsp70/90 – Heat shock protein 70/90

Htt – Huntingtin protein

IAA - Indole-3-acetic acid

IKB – Inhibitor of kB

IKK – IKB kinase

IMAC - Immobilized metal affinity chromatography

iTRAQ - Isobaric tags for relative and absolute quantitation

JNK - c-Jun N-terminal kinases

LB - Luria-Bertani

LMB - Leptomycin B

LOPAC - Library of Pharmacologically Active Compounds

Lys - Lysine

MALDI – Matrix assisted laser desorption/ionisation

MDM2 – Mouse double minute protein 2

MDMX (MDM4) – Mouse double minute protein X (4)

MEK - Mitogen-activated protein kinase kinase

MIRA - Mutant p53-dependent induction of rapid apoptosis

MMTS - Methyl methanethiosulfonate

mRNA – Messenger RNA

MS – Mass spectrometry

MS/MS – Tandem MS

Mt- Mutant

NFAT1 - Nuclear factor of activated T-cells

Nf-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NF-Y – Nuclear transcription factor Y

NIK - NF- B-inducing kinase

NLS – Nuclear localisation sequence

Noxa - Pro-apoptotic member of the Bcl-2 protein family

$P14^{ARF}$/ARF - Alternate reading frame product of CDKN2A locus

p21 – Cyclin dependent kinase inhibitor

p53 – Tumour suppressor p53

p53AIP1 - p53-regulated Apoptosis Inducing Protein 1

PKC – Protein kinase C

PMMA - Polymethylmethacrylate

pp90rsk – p90 ribosomal s6 kinase

PPARS - Peroxisome proliferator-activated receptors

PRD – Proline rich domain

PRIMA – p53 reactivation and induction of massive apoptosis

PTEN - Phosphatase and tensin homolog

Puma - p53 up-regulated modulator of apoptosis

qPCR – Quantitative PCR.

qTOF – Hybrid quadrupole time of flight MS

Rb –Retinoblastoma protein

RETRA - Reactivation of transcriptional reporter activity

RGC – Ribosomal gene cluster

RNA – Ribonucleic acid

RNAi – RNA interference

RPMI - Roswell Park Memorial Institute 1640 medium

RPL11 – Ribosomal protein - Large subunit - 11

RPS3 - Ribosomal protein – Small subunit -3

RPS4X - Ribosomal protein – Small subunit – 4 -X (chromosome)

RPS9 - Ribosomal protein – Small subunit – 9

LC – Liquid chromatography

SCX – Strong cation exchange

SB - SB-415286

SDS - Sodium dodecyl sulphate

SDS-PAGE – SDS polyacrylamide gel electrophoresis

Ser - Serine

SILAC - Stable isotope labelling by/with amino acids in cell culture

siRNA – Short interfering RNA

SirT1/2 - Sirtuin (Silent mating type information regulation 2 homolog) 1/2

SKI – v-ski avian sarcoma viral oncogene homolog

SKIP2 - SKP1/ASK1 Interacting Protein 2

SMAD-2 - Mothers against decapentaplegic homolog 2

SNP – Single nucleotide polymorphism

Src - Proto-oncogene SRC, Rous sarcoma

SUMO - Small Ubiquitin-like Modifier

TAD – Transactivation domain

TCEP - Tris(2-carboxyethyl)phosphine

TD – Tetramerisation domain

TFA - Trifluoroacetic acid

TGF-β – Transforming growth factor - beta

TGFβ-RII – TGFβ receptor 2

TOF – Time of flight

TSP1 – Thrombospondin-1

TWIST - TWIST homolog of drosophila

Ubc9 - Ubiquitin carrier protein 9

UTR – Untranslated region

VEGFR - Vascular Endothelial Growth Factor Receptors

Wt- Wild-type

XIAP - X-linked inhibitor of apoptosis protein

XPC - Xeroderma pigmentosum, complementation group C

Chapter 1

Introduction

1. Introduction

1.1 The molecular basis of Cancer

Cancer is one of the western world's biggest killers with approximately 1 in 3 people in the UK getting cancer at some point in their lives (*1*). Cancer cells have undergone significant uncontrolled changes resulting in cells which have abnormal morphology, altered metabolism, the ability to evade cell death, insensitivity to anti-growth signals and alterations in proliferation (*2*). The exact mechanism by which a cell becomes malignant is unknown but the prevailing model suggests that cells will accrue mutations over time which cause these changes (*3*). It is likely that one of the early events would be the loss of genomic stability which would thus allow mutations and other larger scale genomic derangements to occur at a faster rate (*3*).

Cancers are also frequently linked to carcinogens, chemicals known to be cancer causing which have usually been found to cause damage to the DNA. In fact it can be argued that all cancers will have been caused by long term exposure to carcinogens but that we simply do not know all of these damaging agents and thus are unable to make the link (*4*). Carcinogens or other stressors lead to mutations in a number of exposed cells, if one of these mutations proves a selective advantage e.g. results in faster proliferation, then that cell will multiply at the detriment of other normal cells. A single mutation will not cause a cell to become cancerous however the increased number of cells carrying a mutation increases the chance that one of these cells could accrue further mutations which again may provide a selective advantage. This is called clonal selection, a concept first proposed in 1976 (*5*).

Whilst there are many genes which may become mutated in cancer there are some specific genes which are frequently seen to be mutated in pre-cancerous cells which are thought to be important in the initiation of malignancy. These are frequently genes which are known to be tumour suppressors and include the gene p53 (*6, 7*). Tumour suppressor genes produce proteins which have roles in preventing a cell from becoming cancerous. They act directly or indirectly to ensure

DNA fidelity and typically they are involved in repression of the cell cycle, repair mechanisms and death mechanisms. Loss of these functions can allow the survival and replication of cells with abnormalities and thus allow tumourigenesis.

1.2 The p53 pathway

p53 is one of the best studied tumour suppressors in cancer. p53 is important in mediating a variety of functions including cell cycle control, DNA repair, apoptosis and angiogenesis (*8*). It is activated by a number of stressors and ultimately acts to repair or kill damaged cells thus preventing abnormal cells from continuing to divide (*8*). The importance of this tumour suppressor can be highlighted by the fact that this gene is mutated in around 50% of cancers and its signaling pathway disrupted in many more (*8*).

p53 mediates it's outcomes by both transcriptional and transcription independent mechanisms (*8, 9*). p53 is however primarily thought of as a transcription factor; it binds to p53 response elements in the promoter region of specific genes leading to the up or down regulation of those genes (*8*). The severity of the initial stressor plays an important part in determining the subset of p53 responsive genes which are regulated and thus determining the biological endpoint of activation (Arrest, repair or death) (*10*). Examples of these are shown in Fig 1.1. Exactly how p53 is able to choose between its different outcomes is not entirely clear but may be, at least partly, mediated by the different binding affinities between p53 and its response elements (*11*).

p53's transcription independent functions have only more recently been realized but have been shown to contribute to its role in apoptosis (*9*). Mitochondrial and cytoplasmic accumulation of p53 has been shown to lead to transcription independent apoptosis(*9*). p53 activation appears to be able to contribute to the intrinsic mitochondria-mediated pathway of apoptosis without altering gene transcription (*9*). The initiation of this pathway is usually reliant upon Bcl-2 family proteins. Multiple mechanisms of p53 activation of this pathway have

been put-forward and many suggest that p53 can directly or indirectly activate the proapoptotic Bcl2 family members; Bax and Bak (*9*).

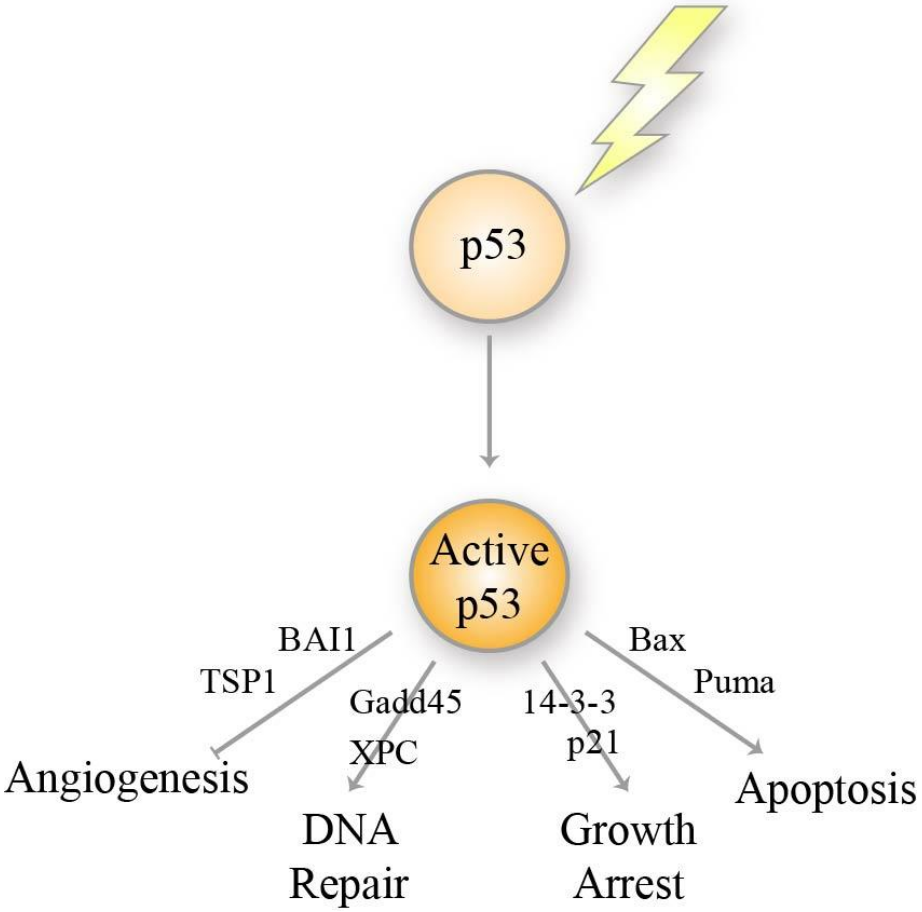

Fig 1.1 Various stress signals can lead to the activation of p53. Active p53 causes the up or down regulation of a subset of p53 responsive genes. Depending on the genes regulated, activation of p53 can lead to a number of different outcomes including DNA repair (*12*), growth arrest (*12*), apoptosis (*13*) or reduced angiogenesis (*14*) (examples of genes involved are shown).

1.3 Regulation of p53

In the body p53 is usually kept inactive in the vast majority of cells (Fig 1.2 top panel), however when a cell experiences stress such as DNA damage, oncogene activation or hypoxia p53 must become active in order to mediate its effects (Fig 1.2 bottom panel). p53 is not thought to be heavily regulated at the transcriptional levels but rather the level of p53 is regulated by its rate of degradation (*8*). The degradation of p53 occurs via the ubiquitin-proteasome pathway, p53 is ubiquitinated by an E3 ubiquitin ligase which targets it to the proteasome where it is degraded. One of the most important and best understood E3 ligases affecting p53 degradation is MDM2 (*15*). Thus the availability and activity of MDM2 is an important factor in controlling the activity of p53 and is an important factor in keeping p53 inactive in normal healthy cells (*8*). The importance of MDM2 can be demonstrated by the fact that knocking out MDM2 is embryonically lethal in mice, however this can be rescued by also knocking out p53 which results in live births (*16*). The transcription of MDM2 is controlled by p53 which acts to up-regulate it. Thus p53 acts to negatively regulate itself via a regulatory feedback loop (*15*).

The ability of MDM2 to negatively regulate p53 is also controlled by post-translational modifications of both p53 and MDM2 (*8, 17, 18*). Furthermore post-translational modification and availability of co-factors are also important in regulating conformation, localisation and activity of p53 (*8, 17, 18*). Both p53 and MDM2 can interact with a large number of proteins and can be subject to post-translational modification including ubiquitination, acetylation, phosphorylation, sumoylation, and glycosylation (*17, 18*). One example of such post-translational regulation is the acetylation of the C-terminus of p53 by p300/CBP which occurs after DNA damage (*19*). The C-terminus of p53 is known to allosterically inhibit p53 activity, however acetylation of residues in the C-terminus appears to relieve this inhibition and promote p53 activity (*19*). A further example is the modification of MDM2 which will indirectly affect p53 activity, for example sumolyation of MDM2 by ubc9 is thought to increase its stability and increase its ability to inhibit p53 (*20*).

It should be noted that whist MDM2 is generally considered as a negative regulator it has also been shown that MDM2 can bind p53 mRNA and increase its synthesis (*21*). The binding of p53 mRNA to MDM2 also reduces its E3 ligase function. The association between MDM2 and p53 mRNA was shown to be increased upon doxorubicin treatment indicating that the affinity between p53 mRNA and MDM2 may play a role in p53 induction (*21*).

Whilst the interactions of p53 with regulatory factors have been subject to intensive research and a huge number of regulatory proteins (*22*) have been identified further investigation is still warranted. It is apparent that p53 interacts with an enormous number of pathways and that many may yet to be discovered and many others are yet to be fully understood. For example a recent study looking for interactors of drosophila p53 yielded 91 previously unreported interactors (*23*). Whilst clearly more work needs to be done to assess these interactions it does demonstrate that our current knowledge of wild type p53 (wtp53) is far from complete. Pathways that repress or activate p53 are potential targets in tumours containing wtp53 so that we can promote activation (see Activation of wtp53). Therefore a fuller understanding will help our efforts to manipulate this pathway.

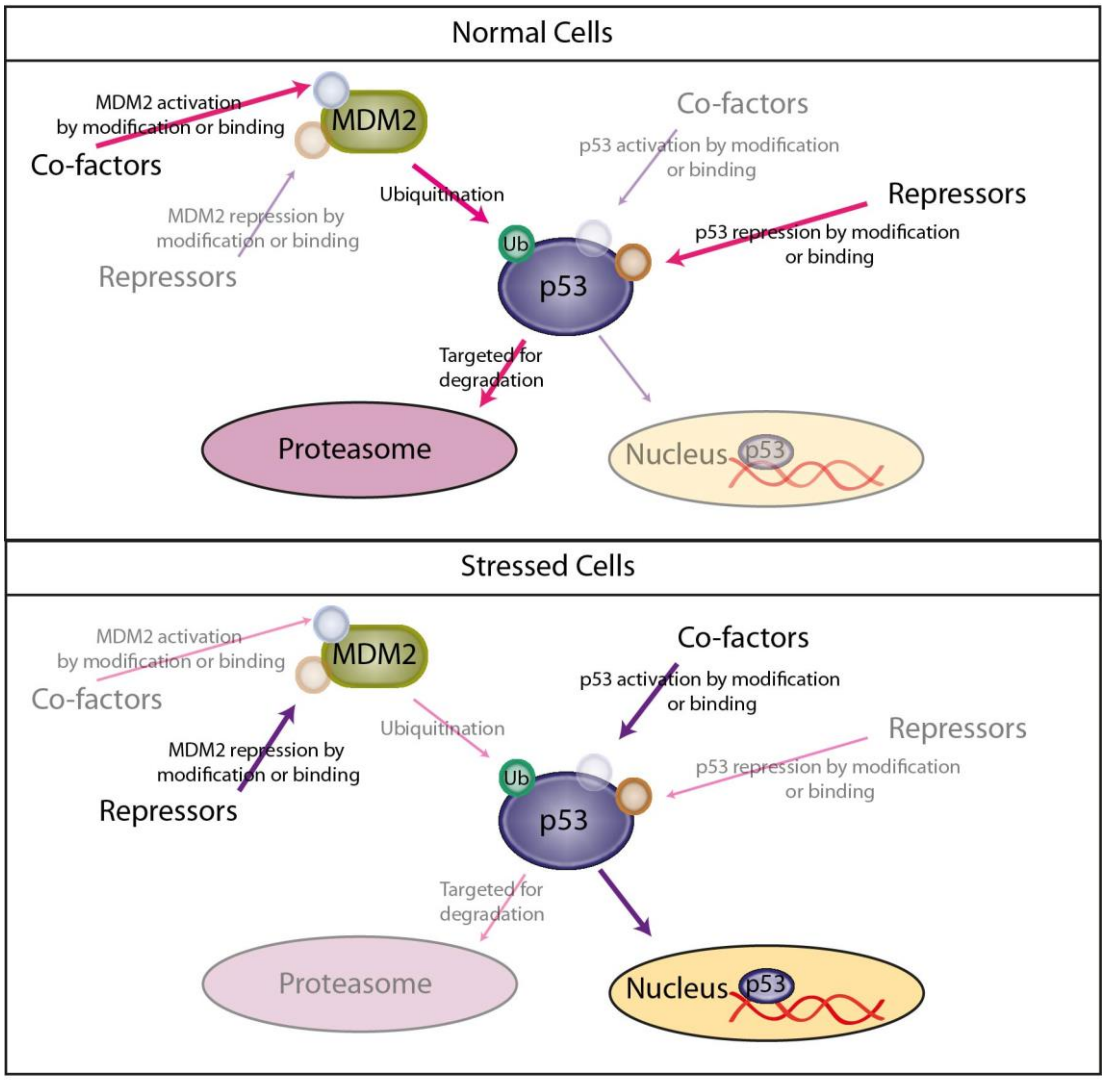

Fig 1.2 Activity and stability of p53 in stressed and unstressed cells. In normal cells p53 is rapidly turned over. Various factors interact with MDM2 and p53 to repress its activity and promote its rapid degradation. MDM2 ubiquitinates p53 directing it to the proteasome. When the pathway is activated by stressors including DNA damage and oncogene activation different factors interact with p53 and MDM2. This represses the ability of MDM2 to target p53 to the proteasome and promotes p53 to enter to nucleus and act as a transcription factor.

1.4 Transcriptional regulation of MDM2

As MDM2 is a major factor controlling p53 its stability and activity is important in determining p53 mediated outcomes (*15*). As mentioned, MDM2 has been shown to be regulated by a large number of proteins, however unlike p53 it is also regulated by transcription as well as post-translational modification (*17, 24*). At the transcriptional level the expression of MDM2 is controlled by two promoters known as P1 and P2 (*24*). The P1 promoter is not controlled by p53 and is thought to be responsible for low level basal MDM2 production (*24*). Control of this promoter is less well understood and research has mainly focussed on the second promoter site; P2. The level of transcription from the P2 promoter is thought to be around 6 times that at the P1 promoter (*25*). The P2 promoter contains two p53 binding elements and is highly inducible in response to p53 and thus is imperative to the negative feedback mechanism (*24*). However, it is apparent that the levels of MDM2 are controlled by other mechanisms than just p53. High levels of MDM2 can be observed in cancer cells without functional p53 (*26*) and research is revealing that there are a number of different response elements within the P2 promoter. For example it has been shown that the P2 promoter contains an NFAT1 consensus site and binding of NFAT1 at this site can enhance MDM2 production (*27*).

1.5 Mutations in p53

Mutation of the p53 gene is one of the most common mutations found in cancer and is often an early event in carcinogenesis (*6, 7*). p53 null mice develop tumours with an exceptionally high frequency (*28*) showing its importance as a tumour suppressor. Additionally germ line mutation of p53 has been shown to be responsible for Li Fraumeni syndrome. Li Fraumeni syndrome is an autosomal dominant disorder characterized by a high susceptibility to cancer. Patients with Li Fraumeni have a mutation in one copy of the p53 gene meaning that only one mutational event is required to entirely lose wild-type p53 activity.

Unlike most tumour suppressors, mutations of p53 tend to be point mutations (*29*). These mutations confer loss of normal function in a variety of ways. The most common mutations are in so called hot-spot regions of the p53 DNA binding domain (Fig 1.3). These either mutate residues which directly interact with the DNA thus preventing binding or cause the protein to lose thermo-stability, this means p53 loses its normal conformation and therefore the availability of its interacting domains is compromised (*29*). These mutations thus reduce the affinity of p53 to its response element thus reducing or removing the ability of p53 to bring about the up regulation of required genes.

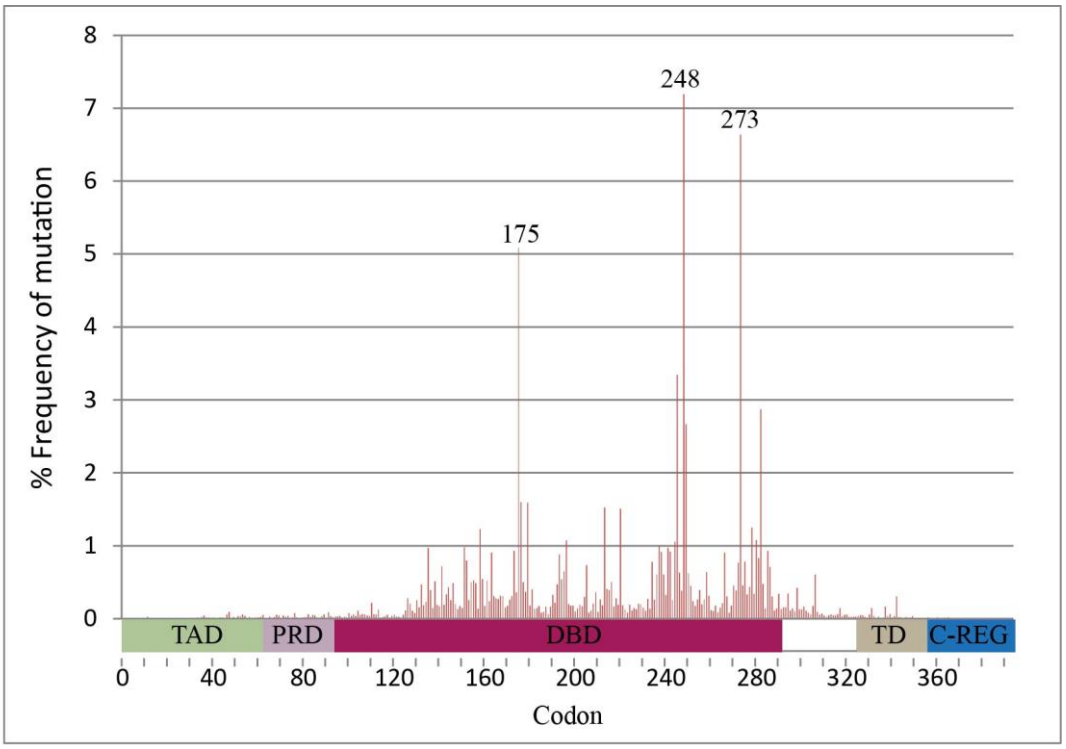

Fig 1.3 Frequency and location of p53 mutations (Data extracted from p53.free.fr). The domains of p53 shown are the transactivation domain (TAD), the proline rich domain (PRD), the DNA-binding domain (DBD), the tetramerisation domain (TD) and the C-Terminal regulatory domain (C-REG).

1.5.1 Oncogenic potential of p53

Mutation in p53 has been shown not only to cause loss of tumour suppressor phenotype but also lead to oncogenic gain of function (*22*). Most mutations in p53 are point mutations, whereas many other tumour suppressors are silenced by deletions (*29*). This has been suggested as evidence that gain of function rather than silencing is being selected for, however it has also been countered that the number of point mutations is not more than expected by chance and p53 may simply be more sensitive to silencing by this mechanism (*29*). Correlative evidence shows that the presence of p53 mutations does indicate a poorer outcome in cancer patients and that certain mutations may lead to poorer outcomes than others (*30, 31*). Evidence from the laboratory supports the idea that gain of function may contribute to this; it has been shown that the introduction of p53 with specific mutations into p53 null cells can cause an increase in chemoresistance and so the presence of particular p53 mutations may lead to a poorer response to chemotherapy (*32*).

The oncogenic capacity of mutant p53 (mtp53) can be attributed to both its dominant negative and gain-of-function capacities. It has been demonstrated that when mtp53 is expressed in a cell with wtp53, mtp53 represses the transcriptional activity of wild-type p53 (*33*). p53 naturally functions as a tetramer and it has been suggested that the binding of mtp53 to wtp53 within the tetramer will inhibit wtp53 activity (*33*). mtp53 has also been noted to be able to inhibit its family members; p63 and p73 (*34*). p63 and p73 are also tumour suppressers capable of regulating the expression of many p53 regulated genes, although knockout experiments have shown that the functions are non-redundant (*35*). p53 with certain mutations have been shown to bind to p63 and/or p73 leading to negative regulation (*34*). The interaction appears to be mediated through the DNA binding domains and it has been shown that the denatured DNA domain of p53 is capable of binding the DNA binding domain of both p73 and p63, preventing them from binding their target DNA (*36*).

Some of the observed "gain of function" effects could also be the result of mtp53 activating some of its normally regulated genes but not others. Pan and Haines (2000) showed that p53-R213Q was capable of promoting the transcription of MDM2 but not factors important in cell cycle arrest or apoptosis (*37*).

In addition to repressing wtp53 and related family members, mtp53 has been described to have many other abnormal functions. Mtp53 like wild-type p53 appears to be able to promote or repress a number of genes (*38*). However wtp53 and mtp53 appear to regulate a distinct subset of genes. Whilst wtp53 dependent gene expression promotes apoptosis and cell cycle arrest certain gain-of-function interactions confer alterations including enhanced survival, proliferation and metastasis (*38*). It is becoming clear that mtp53 can interact with a number of factors that wtp53 does, however the outcomes are often different (*38*).

The mechanism of how mtp53 controls transcription is unclear in many cases but some examples have shown that mtp53 can aberrantly interact with certain transcription factors and thus this could provide a mechanism of how mtp53 could achieve sequence specific binding (*38*). Examples of signalling pathways altered by mutant p53 including Nf-κB, EGR-1, TGF-β, and NF-Y will be discussed (summarised in Fig 1.4). These examples however are by no means exhaustive.

Nf-κB is known to be an important factor in cancer and furthermore dysregulation in the cross-talk between the Nf-κB pathways and p53 pathways has been shown to contribute to oncogenesis (*39*). In normal tissues it has been shown that depending on the circumstances p53 and Nf-κB can work together to be pro-apoptotic or work against each other with Nf-κB acting as a pro-survival factor (*40*). When p53 is mutated however it appears to act to promote Nf-κB via increased transcription of the p100/p52 Nf-κB subunit (*41*). This increase in Nf-kB enhances its activity which contributes to the cells survival and drug resistance (*41*). This is of particular interest as it has also been shown that mutation in p53 correlates with constitutive Nf-κB pathway activation (*42*).

It has also been shown that mutant p53 can lead to the increased production of EGR-1 (*43, 44*). EGR-1 is a transcription factor important in controlling apoptosis

and growth, and its aberrant production has been shown to enhance survival in some cancers (*45*). Initial reports suggested that mtp53 directly binds to the EGR-1 promoter (*43*) however more recent data suggests that mtp53 does not directly bind the promoter but enhances it activity by initiating a complex feedback loop involving EGFR and MEK signalling (*44*). The apparent reason for these discrepancies remains unclear although it is possible that different mechanisms are important in different cells or with different p53 mutants. Nonetheless EGR1, like many of the proteins affected by mutant p53, may represent a useful drugable target. Evidence shows that reduction of EGR1 by siRNA reduces tumour cell growth and increased apoptosis (*43*).

The interaction between mtp53 and the TGF-β pathway is more complex and appears to be at least partially dependant on tumour stage and type. TGF-β is thought to act as a tumour suppressor although research has shown that this role may be more relevant in early tumourigenesis (*46*). Evidence has shown that mutant p53 can suppress TGF-β signalling consistent with the idea that mtp53 is tumourigenic and TGF-β signalling acts as a tumour suppressor (*47*). The pathway was shown to be repressed via reduced expression of TGFβ-RII as a result of p53 binding to the promoter region (*47*). However another study has shown that mutant p53 may co-operate with the TGF-β pathway by binding to SMAD-2 and promoting its suppression of p63. This results in the attenuation of metastasis suppressor genes which are under the transcriptional control of p63 (*46*). This mechanism may be more important in late stage cancers and enhance a metastatic phenotype (*46*).

Many of the above examples propose that mtp53 can regulate gene transcription however, in many cases, the mechanism by which this can occur is not entirely clear. The binding of mtp53 with transcription factors provides a mechanism of how mtp53 could selectively regulate certain genes. One example of mtp53 interacting with a transcription factor is with NF-Y (*48*). When DNA damage occurs in cells with wt-p53 various genes including cyclin A and cyclin B1 are downregulated to prevent cell cycle progression. This transcriptional alteration is dependent upon a complex of both NF-Y and wt-p53 which leads to histone

deacetylases being recruited (*48*). Reduced acetylation of histones results in the DNA being more tightly wound and reduces transcription from that area. Mutant p53 has also been shown to bind to NF-Y however upon DNA damage this conversely leads to the up-regulation of these genes resulting from p300 histone acetyltransferase recruitment (*49*). Thus this binding results in aberrant activation of NF-Y genes and deregulation of the cell cycle. This is a particularly important example of mtp53 transcriptional activity as it provides at least a partial mechanism by which mtp53 is functioning.

Further work will be needed to resolve the mechanism by which many proposed mtp53 responsive genes are controlled. No mtp53 consensus DNA binding site has been identified but clearly binding of mtp53 to other transcription factors provides it with site specific activity. It has also been suggested that structure specific binding may play a role in determining the mtp53 response (*38*) however again further research is needed to resolve the importance of this.

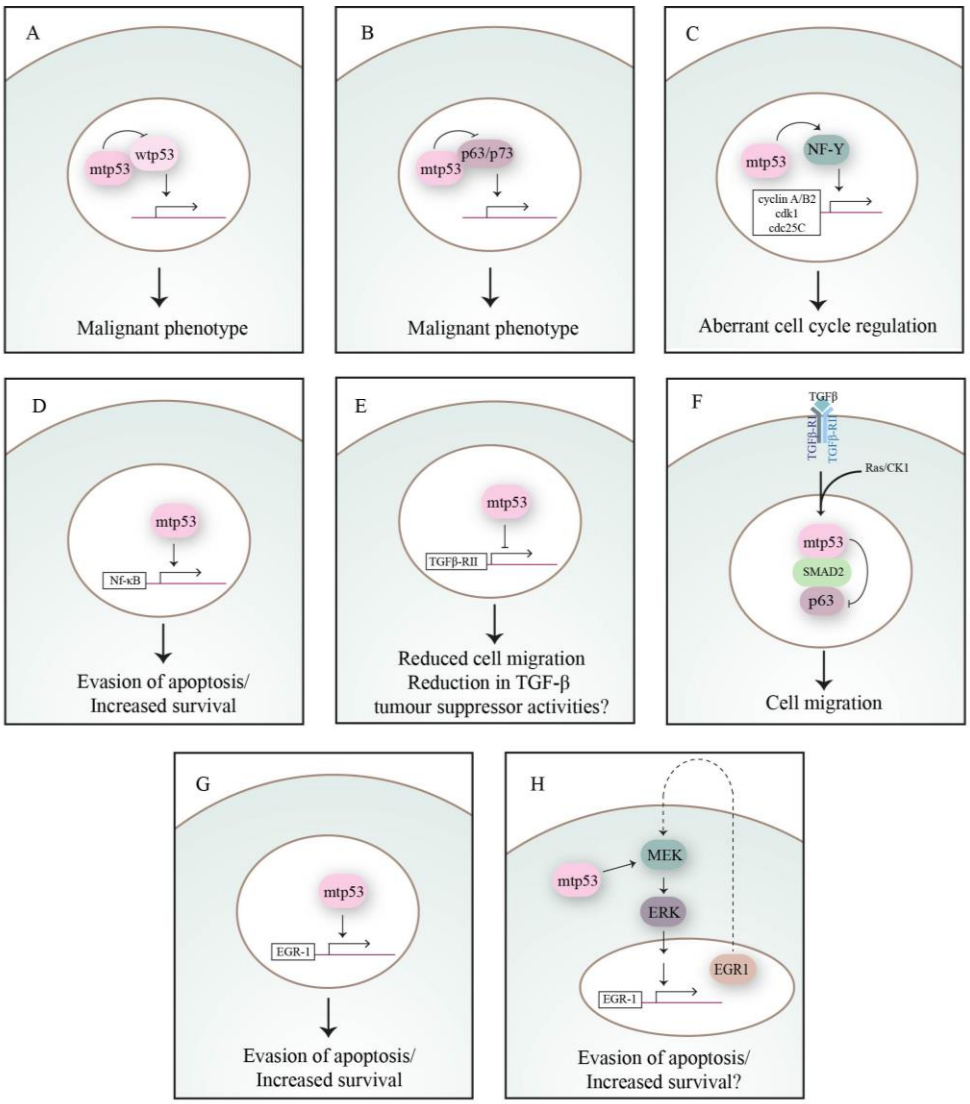

Fig 1.4 Potential mechanisms of the pro-oncogenic function of p53. Figure adapted from Maslon and Hupp (2010) **(A)** mtp53 binds to wtp53 suppressing the wtp53 function. This acts to promote the malignant phenotype. **(B)** mtp53 directly binds p63 or p73 thereby repressing their ability to induce the expression of p53 pathway genes. This acts to promote the malignant phenotype. **(C)** mtp53 enhances the expression of Nf-κB. This results in evasion of apoptotic signals. **(D)** mtp53 co-operates with NF-Y to enhance the expression of genes important in cell cycle progression. This results in aberrant regulation of the cell cycle. **(E)** mtp53 suppresses the expression of TGFβ-RII. This results in reduced cell migration but potentially allows cells to escape TGF-β tumour suppressor effects early in tumourigenesis. **(F)** TGF-β and Ras-CK1 signalling cooperate in enhancing the binding of mt-p53 and SMAD2 which represses p63 activity. This results in increased cell migration. **(G+H)** Two mechanisms of how mtp53 increases EGR-1 which results in evasion of apoptotic signals have been proposed: **(G)** mtp53 directly enhances the expression of EGR-1. **(H)** mtp53 promotes MEK activity leading to the downstream transcription of EGR-1. EGR-1 then indirectly acts to increase MEK signalling which promotes EGR-1 production and thus mtp53 acts to initiate a feedback loop.

1.6 Regulation of mtp53

As discussed mtp53 has altered interactions with various proteins in comparison to wtp53, such changes are important in its pro-oncogenic capacity. Similarly the mutant protein is also subject to different regulation and most notably mtp53 is much more stable in terms of its half-life than wtp53 in cancer cells (*50, 51*). Evidence suggests that the proteins involved in the degradation of mtp53 may be different in comparison to wtp53 (*50, 51*). Some changes in regulation may result intrinsically from the alterations to the protein and other alterations may actively be selected for to increase the oncogenic capacity of the mutant protein. Understanding the pathway involved in mtp53 stability and degradation are of particular importance as evidence suggests reduction of p53 levels may be therapeutically useful (*52, 53*).

1.6.1 mtp53 stability

Despite the protein not triggering an arrest or apoptotic response, mtp53 accumulates within the cell (*50*). Evidence suggests that this accumulation of p53 may actually be selected for in a cancer cell (*32, 54*). Unlike with wtp53, MDM2 appears to be incapable of directing p53 for degradation (*51*). However, despite this, mtp53 is often still ubiquitinated. In fact mtp53 has been seen to be hyperubiquitinated in comparison to wtp53 and this process may be at least partially dependent on other E3 ligases (*50*). As discussed mutation in p53 may confer a number of oncogenic gains of function and so an increased level of mtp53 should be advantageous to the tumour cells. As such, bringing about the degradation of this mutant protein may provide a clinically desirable effect.

The simplest explanation of high levels of mutant p53 would be that due to its inability to bind to the DNA response element, MDM2 cannot be expressed and therefore p53 cannot be degraded. However high levels of mtp53 are sometimes concomitant with high levels of MDM2 (*26*). Furthermore Terzian et al. (2008)

showed that in mtp53 mice, normal tissues retained very low levels of p53 and it is only in tumours that high p53 levels are detectable (*54*). It was shown that in these normal tissues expressing mutant p53 that degradation of p53 is still at least partially MDM2 dependant. It should also be noted that low levels of mtp53 in normal tissues also precludes the idea that it is any other intrinsic attribute of the p53 mutant that causes its stability. This indicates that there must be further changes in cancer cells which lead to this accumulation of p53. Terzian et al. (2008) also showed that whilst most cancers in the mtp53 mice had increased levels of p53 it was not universally true, showing that this accumulation is also not strictly necessary for the cancer phenotype (*54*).

One mechanism by which p53 could escape MDM2 mediated degradation is by altered cross-talk with the Retinoblastoma (Rb) pathway. The Retinoblastoma protein (Rb) is a tumour suppressor important in cell cycle control. In an unphosphorylated form it binds to, and represses, the transcription factor E2F1 which normally acts to promote cell cycle progression (*55*). Terzian et al. (2008) showed that if p53 alone was mutated then normal tissues retained normal p53 levels. However if $p16^{INK4a}$, a protein in the Rb pathway, was also lost then normal tissues had high levels of p53. There is significant cross-talk between the Rb and p53 pathways and defects in the Rb/$p16^{INK4a}$ pathway can lead to hyperactivation of the transcription factor E2F1 which in-turn can result in an increase in $p14^{ARF}$ repressing MDM2 and promoting p53 stability (*55*). Retrospective analysis of tumours derived from mutant p53 mice revealed that a number of these tumours had reduced or showed no expression of $p16^{INK4a}$ or other alterations of other Rb pathway proteins. As members of the Rb pathway, including $p16^{INK4a}$, are frequently found to be altered in cancer (*55*) this may be a mechanism which contributes to p53 stability. However it should be noted that $p16^{INK4a}$ mutation did not lead to p53 stabilisation in all normal tissues. In fact loss of MDM2 could not stabilise p53 in all tissues either. This suggests that the normal mechanisms controlling p53 are not the same for all cell types and so the mechanisms by which cancers achieve high p53 levels are likely to be variable also.

1.6.2 mtp53 E3 ligases

Whilst MDM2 is the best studied and best known p53 E3 ligase it has become clear that a number of other E3 ligases are able to ubiquitinate p53 both in normal and cancer tissues and include Cop1 (*56*), CHIP (*57*) and ARF-BP1 (*58*). It is unclear whether their importance are distinct in terms of cell type, location, developmental stage or other factors, however the embryonic lethality of MDM2 (*16*) suggests at least some degree of distinct function.

The relative importance of each of these in ubiquitinating p53 however may be altered for mutant p53. In general it appears that MDM2 may be less important in the turnover of mtp53 compared to wtp53 in many cancer cells and does not contribute greatly to its ubiquitination (*50, 51*). COP1 has been shown to able to ubiquitinate both wtp53 and mtp53 although any difference in the relative affinities is as yet unclear (*50*). COP1 has been shown to be up-regulated in a number of cancers however this up-regulation has been shown to be associated with low p53 and so may be more important when wtp53 is present (*59, 60*). ARF-BP1 was identified as an ARF binding proteins which is known to repress MDM2 activity and promote p53 activity (*58*). ARF-BP1 was shown, at least in certain cases to ubiquitinate only wt p53 and does not appear to be important in mtp53 ubiquitination (*50*). CHIP, however, is thought to be particularly important in the regulation of mtp53 although it is also capable of wtp53 degradation (*50, 51*). Lukashchuk et al. (2007) showed that if MDM2, ARF-BP1, COP1 and CHIP were silenced only COP1 and CHIP silencing obviously decreased mtp53 ubiquitination, with CHIP having the most substantial effect (*50*).

Whilst mtp53 is ubiquitinated, at least by COP1 and CHIP, it is not efficiently degraded. One factor which may be important in protecting mtp53 from degradation is Hsp90. Hsp90 is a chaperone molecule that has been shown to interact with p53 (*61*). Hsp90 interacts with a large number of proteins and is thought to be important in protein folding (*62*). Under stress conditions hsp90 interacts with a large number of proteins and is important in maintaining their

integrity and appears to holds true for a number of proteins in cancerous cells (*62*). Muller et al. (2008) found that mtp53 types with a more folded conformation appear to have longer half-lives and are dependent upon hsp90 for sustaining this conformation (*51*). The authors showed that inhibiting Hsp90 using the compound 17-AAG led to an increase in the amount of p53 bound to hsp70 as well as increased p53 degradation of by the E3 ligase; CHIP. This again indicates that CHIP is one of the p53 E3 ligases best able to recognize p53 in its mutant form. Promotion of this activity may therefore be a useful therapy and 17-AAG has been pursued as a potential chemotherapeutic (*62*). Although it should be noted that the use of 17-AAG is not restricted to cells with mutant p53 as it is important in the regulation of various oncogenic proteins.

1.7 Mutations in MDM2

Whilst mutation of p53 is a relatively common event mutation of MDM2 is rarely seen. Nonetheless a few cases of MDM2 mutation have been observed in cancer; Schlott et al. (1997) found mutations within the zinc finger region in a number of tumours (*63*). Lindström et al. (2007) later showed that these mutants have impaired ability to promote the degradation of p53 but retain the ability to repress p53 transcriptional activities (*64*). They also showed that such mutations in MDM2 prevented the binding of ribosomal genes including RPL11. RPL11 is thought to have a role in preventing MDM2 mediated repression of p53 during ribosomal stress thereby inducing cell cycle arrest. It was postulated therefore that these mutations could help tumours become insensitive to anti-growth signals generated by ribosomal stress (*64*).

A single nucleotide polymorphism within the MDM2 promoter region has also been shown to be relevant in cancer susceptibility. SNP309 in the promoter of MDM2 has been shown to lead to enhanced binding of the SP-1 transcription factor leading to greater transcription and higher protein levels (*65*). SNP309 has been identified to be associated with earlier onset cancer (*65*) presumably as a result of p53 repression and other oncogenic abilities.

In view of the fact that MDM2 is largely considered to be oncogenic it is unsurprising that inactivating mutations are not generally observed. Whilst mutation of the MDM2 gene is thought to be a relatively rare event, MDM2 gene amplification in cancer is relatively frequently observed and is associated with protein overexpression (*26, 66*). This will be discussed below (See mechanisms of MDM2 overexpression).

1.8 Dysregulation of wtp53 and MDM2

As discussed, the loss of p53 activity is thought to be crucial to cancer progression and the frequency of p53 mutation is very high. Despite this a number of tumours retain wt p53, and p53 mutation is in fact a relatively uncommon event in some cancer types (*67-69*). However, in such cancers p53 activity is generally thought to be repressed by alternate mechanisms. As discussed, amplification of MDM2 is a relatively common occurrence in cancers and is thought to act to prevent p53 activation (*70, 71*). Other mechanisms of p53 activity repression have also been identified and include direct inhibition of p53 or promotion of MDM2 activities (*72-75*).

1.8.1 MDM2 overexpression

MDM2 overexpression is a relatively frequent event in cancer and gene amplification is seen in many, but not all, cases (*26*). Alternative potential mechanisms of MDM2 overexpression include increased transcription, translation or stability changes. The overexpression of MDM2 results in inhibition of p53 which prevents its activation and promotes cell survival (*76, 77*). Interestingly, mutation of p53 is rarely seen alongside MDM2 gene amplification (*26, 66*) presumably as the major pressure for either genetic derangement is repression of wtp53. However in some cases MDM2 amplification and p53 mutation are seen together (*26, 66*), this suggests that both MDM2 and p53 mutation can independently offer some increased oncogenic abilities.

MDM2 can also be found to be overexpressed in the absence of gene amplification (*26*). The mechanisms by which such alterations actually occur in cancers are not clear however some potentially important factors are discussed.

One potential mechanism is the alteration of MDM2 stability; a number of factors which are able to effect the degradation of MDM2 are altered in cancer. An example of this is β-TrCP1. MDM2 is able to auto-ubiquitinate, however it has also been shown that it can be ubiquitinated by the E3 ligase β-TrCP1 (*78*). Ubiquitination by β-TrCP1 requires MDM2 to be phosphorylated at multiple sites; a process that requires CK1 (*78*). β-TrCP1 has been observed to be mutated in some cancers however overexpression is seen in others, leading to speculation as to whether β-TRCP is a tumour suppressor or an oncogene (*79*). Nonetheless it remains possible that altered activity within the β-TRCP pathway in certain cancers represents a mechanism of MDM2 dysregulation.

An alternative pathway affecting MDM2 stability is interaction with SKI. SKI is an oncogene which has found to be overexpressed in a number of cancers (*80*), one proposed mechanism of action is its ability to increase MDM2 levels (*81*). SKI interacts with Ubc9, a SUMO conjugating E2, which leads to the increased sumolyation of MDM2 which enhances its stability (*81*). However, once again, the actual contribution of this pathway to p53 repression needs to be further explored in cancers.

A number of studies have shown that some patient samples contain overexpression of MDM2 concomitant with increased MDM2 mRNA but without gene amplification (*26*). One potentially important mechanism for increased transcription relies on the NFAT1 transcription factor. NFAT1 has been shown to increase MDM2 transcription from the P2 promoter (*27*). NFAT1 has been shown to be overexpressed in certain cancers and this overexpression correlates with MDM2 overexpression (*27*).

Finally, it has also been shown that an increased rate of translation may also be important in MDM2 overexpression. Landers et al. (1997) showed that the MDM2 increase in a number of melanoma cell lines resulted from relatively small

increases in mRNA production from the P2 promoter (*25*). In normal cells expression from the P2 promoter is thought to be more important in induced expression as it is p53 responsive (*24*). The authors showed that the transcript produced had a shorter 5'UTR and 3-6 fold increased translation in comparison to the transcript from the P1 promoter (*25*). This means that which transcript is produced will also affect the rate of translation.

1.8.2 Other mechanisms preventing p53 activity

Whilst MDM2 overexpression is a relatively common occurrence in wtp53 cancers, not all wtp53 cancers show overexpression of MDM2 and a number of other mechanisms by which p53 can be repressed have been identified. In some cancers the MDM2 family protein MDMX is also found to be overexpressed (*73*). MDMX appears to be a negative regulator of p53 however it has not been shown to have appreciable p53 E3 ligase activity. Nonetheless its activity as a negative regulator of p53 is exemplified by the fact that MDMX knockout is embryonically lethal but is rescued by p53 knockout (*82*). MDMX appears to act on p53 directly by binding and blocking the transactivation domain (*83*) but also indirectly by stabilising MDM2 (*84*). Furthermore, research has shown that overexpression of MDMX can inhibit the activation of p53 by the MDM2 inhibitor Nutlin and therefore suggests that MDMX may be an important potential target in certain cancers (*85*).

The p53 family protein, p73, is generally thought of as a tumour suppressor gene however it has been shown that it can be overexpressed in cancer (*72*). Research has shown that specifically a splice variant of p73 lacking the transactivation domain ΔNp73 may be overexpressed (*72*). ΔNp73 binds to and inhibits both p53 and full length p73 resulting in enhanced drug resistance and reduced apoptosis (*72*) suggesting that ΔNp73 may be a particularly important factor in cells expressing wtp53.

Loss of expression from the CDKN2A locus on chromosome 9p21 is another relatively common occurrence in cancer (*74, 75*) which has been suggested to impact p53 activity. This gene expresses alternative transcripts which encode the

proteins $p14^{ARF}$ (ARF) and $p16^{INK4a}$ (INK4a)(*86*). INK4a is an inhibitor of cyclin dependent kinases, cdk4 and cdk6. Cdk4 and cdk6 promote cell cycle progression and thus INK4a inhibitory action can lead to cell cycle arrest (*86*). Likewise ARF also acts to inhibit the cell cycle but in a p53 dependent manner. ARF binds to and inhibits MDM2 thus promoting p53 activation (*87, 88*). Silencing of ARF in mice has been shown to lead to them becoming highly prone to tumour development (*89*), however such mice do not succumb to the same spectrum of tumours as p53 knockout mice (*89*) suggesting that p53 independent functions exist. Some studies have shown that MDM2 overexpression and ARF loss tend to be seen in wt-p53 cells (*74*) and that MDM2 overexpression and ARF loss tend to be exclusive (*75*) suggesting that the inhibition of p53 is the reason such changes occur. However, other studies have shown that such events are not exclusive (*90*) and thus suggest that other p53-independent mechanisms of ARF loss may be important for tumourigenesis in some cancers. Nonetheless this does not discount the idea that ARF loss is a major mechanism of p53 inactivation in cancer.

A final example of a potentially important mechanism in repressing p53 is the overexpression of TWIST. TWIST is an oncogene that has been shown to be overexpressed in some cancers and binds to, and inhibits the activity of p53 (*91*). TWIST has been shown to bind to p53 and block its phosphorylation at ser392 which enhances the availability of p53 to MDM2 (*91*).

These examples of mechanisms of p53 activity repression are not exhaustive and it is likely that many mechanisms are yet to be discovered. Identification of such mechanisms is critical and many may represent drugable targets to aid cancer treatment (See *10.10.1 Activation of wtp53*).

1.9 p53 independent roles of MDM2

As discussed MDM2 is frequently overexpressed or misregulated in cancer. This is often considered to occur because of its role in the regulation and degradation of p53. However, whilst p53 mutation and MDM2 overexpression are more frequently not seen together this is not always the case. This hints at the

possibility that other p53-independent roles may contribute to cancer. In is becoming apparent that MDM2 is important in multiple p53 independent pathways. It has been shown that whilst $p53^{-/-}$ mice and $p53^{-/-}$ mice overexpressing MDM2 give rise to tumours with a similar frequency the spectrum of tumours is changed suggesting a p53 independent role of MDM2 in tumourigenesis (*92*). Furthermore MDM2 splice variants which cannot bind p53 have also been seen to be oncogenic (*93*).

MDM2 can affect the cell cycle via its interaction with a number of proteins including Rb family proteins. When Rb is in an unphosphorylated form it binds to, and represses, the transcription factor E2F1 which normally acts to promote cell cycle progression (*55*). There is significant cross-talk between the p53 pathway and the Rb pathway however it appears that MDM2 can have a regulatory role in the pathway independently of p53 (*55*). It has been shown that MDM2 is capable of ubiquitinating Rb and so promotes its degradation. Overexpression of MDM2 therefore represses the tumour suppressor activities of Rb (*94*). The relief of Rb negative regulation of E2F1 also leads to an up-regulation of genes including DNMT3A (*95*). DNMT3A leads to the silencing of genes including tumour suppressor genes by DNA methylation. MDM2 also appears to be able to directly enhance E2F1 activities independently of Rb ; MDM2 binds to E2F1 which reduces the ability of SKP2, the E2F1 E3 ligase, from binding and ubiquitinating E2F1 (*96*).

MDM2 is also able to act as an E3 ligase for the protein Numb; a protein important in cellular differentiation. It is thought to act as a tumour suppressor by multiple mechanisms including reducing the interaction of MDM2 and p53 (*97*) and also through its inhibitory action on notch (*98*). The balance between notch and Numb is important in controlling cell proliferation and the loss of Numb is thought to be particularly important in breast cancer (*98*). Overexpression of MDM2 is thought to reduce the levels of Numb and it has been shown that high levels of MDM2 negatively associate with Numb (*97*).

Additionally, MDM2 has also been demonstrated to be capable of increasing the transcription of the Nf-κB subunits. Nf-κB has numerous functions in the cell

including roles in apoptosis, inflammation, immunity and cancer (*99, 100*). Nf-κB is considered to be oncogenic as it frequently acts as an anti-apoptotic factor (*99*). Gu et al. (2002) showed that increased MDM2 levels lead to an increase in the Nf-κB family protein p65, which increased doxorubicin resistance (*101*). In the presence of p53 MDM2 appeared to relieve negative regulation of p53 on p65. However, in the absence of p53 MDM2 still increased p65 levels and was seen to directly bind to the promoter. Similarly Vaughan et al. (2011) found that MDM2 can lead to the up-regulation of the p100/p52 Nf-κB subunit in a p53 independent fashion which lead to an increase in the transcription of Nf-κB target genes and also enhanced cell survival (*102*).

MDM2 has also been shown to interact with a number of ribosomal subunits. A number of studies have shown that this interaction can inhibit the E3 ligase activity and promote p53 (*103*). It has been suggested that this provides a functional link between ribosomal stress and apoptosis. As mentioned previously MDM2 has also been shown to be capable of binding RNA strands and can positively regulate p53 transcription (*21*). The fact that MDM2 can bind both RNA and ribosome proteins has led to further speculation as to whether MDM2 may have a greater role in transcriptional regulation of other proteins. It has since been found that MDM2 also regulates the transcription of XIAP (*104*). XIAP is an anti-apoptotic protein which is important in survival in stress conditions. Gu et al. (2009) showed that MDM2 is capable of binding the internal ribosome entry site of XIAP and enhance its translation (*104*). This effect was shown to be important in mediating resistance to stress such as ionising radiation.

1.10 Targeting p53

As discussed the p53 pathway is an attractive therapeutic target, however there are multiple ways in which it could be targeted, in part depending on the p53 status of the cell. If wtp53 is present in the cell then therapy can aim to enhance its activation. If mtp53 is present therapy can aim either to reactivate p53, in order to bring about wild-type function, or alternatively abolish any oncogenic gain of

activity the protein may have. Targeting the oncogenic p53 function could be achieved by two different methods, either such functions could be identified and specifically targeted or degradation of mtp53 could be enhanced in order to reduce all potentially oncogenic activities. The various ways of targeting wtp53 and mtp53 are summarised in Fig 1.4 and a number of examples are discussed below.

The different approaches to p53 targeting highlights the requirement to know the mutational status of p53 in the cancer. For example administering a therapy which activates p53 would be useful for patients with wtp53 cancers but could be useless or potentially detrimental to those harbouring p53 mutations.

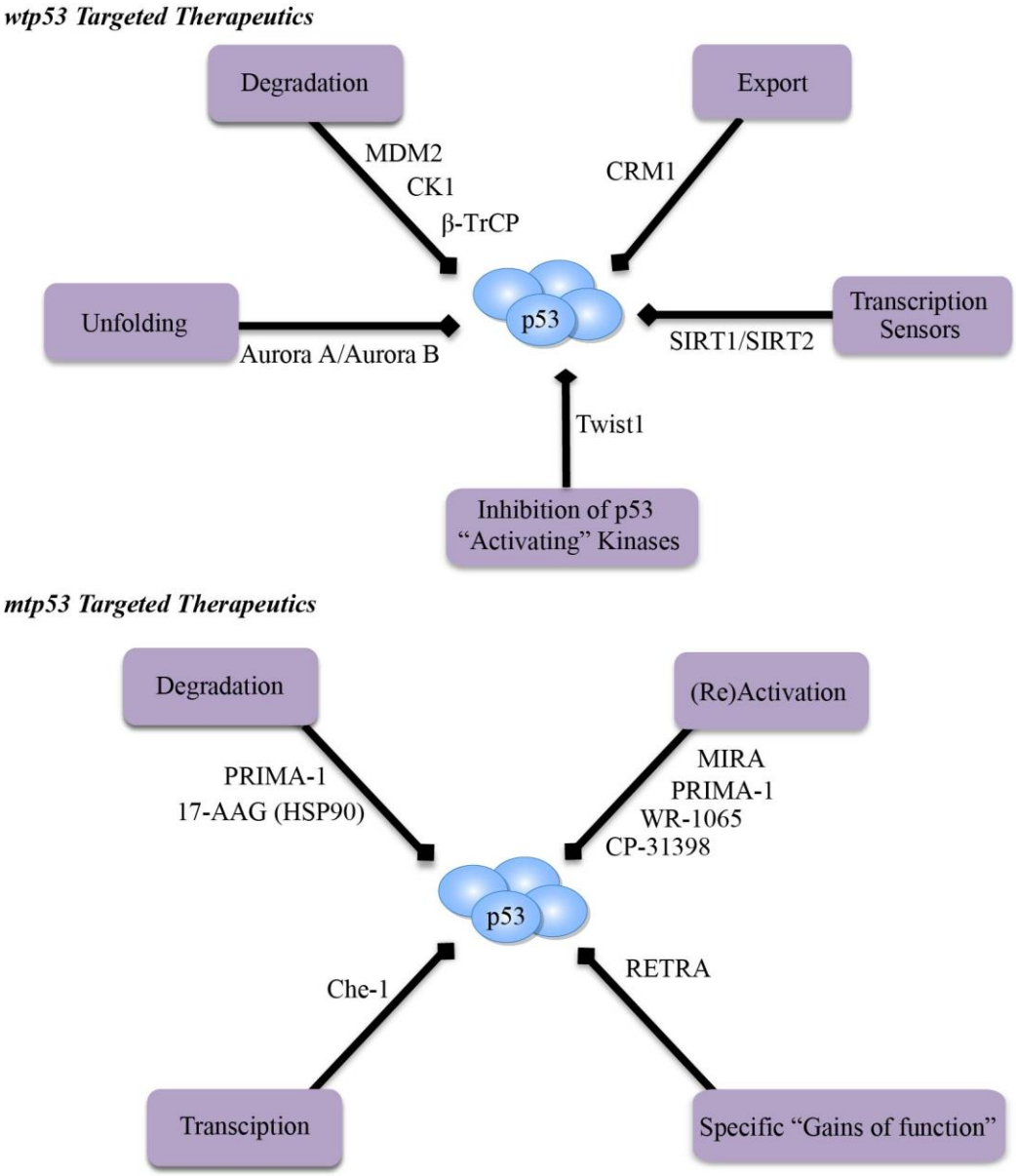

Fig 1.5. Targeting wtp53 and mtp53 (Fig modified from Hupp et al. (2012) (*105*)). Wtp53 can be targeted by manipulation of factors controlling activation, transcription, unfolding, export and degradation. Mutant p53 can be targeted by promotion of wild-type function (Reactivation), removal of protein (Degradation, Transcription) or inhibition of specific gains of function. Examples of potential targets or drugs to manipulate these factors are shown.

1.10.1 Activation of wtp53

Many cancers retain wtp53 and there are a number of cancer types where p53 is rarely mutated (*67-69*). It has been suggested that direct p53 activation should be more harmful to cancer cells than normal cells and some studies have shown that activation is more likely to causes apoptosis in cancer and cell cycle arrest in normal cells (*106, 107*), however this will likely be dependent on the particular mechanism utilised. As p53 interacts with such a large number of other proteins it could potentially be activated by a number of mechanisms. Therapy could potentially seek to alter p53 stability, subcellular localisation (export), transcriptional abilities, folding or the activity of p53 activating kinases (Fig 4). Examples of compounds or targets affecting these different mechanisms will now be discussed and the structures of these compounds are detailed in Fig 1.6.

The stability of p53 could be increased by targeting factors involved in the degradation of p53. Inhibiting the activity of MDM2 is thought to be a particularly attractive option as MDM2 is overexpressed in many cancers (*26*). A number of MDM2 inhibitors have been developed but the furthest advanced is Nutlin. Nutlin-3 is the most commonly used variation of this drug and its structure is shown in Fig 1.6. The Nutlin compounds mimics 3 residues within the transactivation domain of p53 and thus competitively inhibits the binding of p53 with MDM2 (*108*). At least within cancer cells inhibition of MDM2 appears to be sufficient to activate p53 and Nutlin was shown to lead to p53 dependant apoptosis and cell cycle arrest in cancer cells. Furthermore in studies on mice with human tumour xenographs treatment with non-toxic doses of Nutlin lead to tumour shrinkage (*108*). Nutlin is now in early stage clinical trials (*109*).

An alternative way to increase the stability of p53 is inhibition of the proteasome. Clearly this is a less specific therapy which would affect proteins outside the p53 pathway however a number of proteasome inhibitors are being considered for, or used as, cancer therapeutics. This includes Bortezomib which is currently used in the treatment of multiple myeloma and mantle cell lymphoma (*110*). These drugs are thought to work because a number of tumour suppressor

proteins are removed from cancer cells due to rapid turnover by the proteasome system, thus inhibition of this system will allow these proteins to accumulate and inhibit or kill the cancer cell (*110*). Clearly this approach is relatively non-specific but data supports the fact that Bortezomib causes apoptosis in cancer cells (*110, 111*). Studies have shown that Bortezomib does lead to the accumulation and activation of p53 (*111*) however studies have found that cell death in some cancers appears to be independent of p53 (*112, 113*). This may be because other accumulating pro-apoptotic factors in certain cells are able to more rapidly induce apoptosis.

Altering subcellular localisation has also been seen to influence p53 activity. In order to be transcriptionally active p53 needs to be located to the nucleus and nuclear export can prevent its activation. The compound Leptomycin B (LMB) which inhibits the nuclear export protein CRM1 has been shown to increase the activity of p53 (*114*). Again, it should be noted that CRM1 inhibition will also affect a number of other proteins which could contribute to its activity. Inhibition of CRM1 prevents the degradation of p53 and sequesters p53 in the nucleus which is required for its activity (*115*). LMB showed significant toxicity *in vivo*, however newer nuclear export inhibitors are being developed which have lower toxicities. For example Mutka et al. (2009) showed that a LMB derivative lead to apoptosis in cancer cells but cell cycle arrest in normal cells and additionally was shown to have an anti-tumourigenic effect *in vivo* in a mouse model (*116*).

A further example of a potential therapeutic which activates p53 is Tenovin; Tenovin was discovered via a screen of small molecules using cells with a p53 responsive β-gal reporter construct (*117*). Fig 1.6 shows the structure of Tenovin-6 a more water-soluble and therefore more easily administered version of the original Tenovin molecule (*117*). Characterisation showed that Tenovin leads to p53 activation and cell death in culture and reduced tumour growth *in vivo* (*117*). This effect was suggested to be promoted by p53 activation but it was shown that p53 activity was not essential as Tenovin could kill cells with mtp53 or no p53 (*117*). The target of Tenovin was shown to be SirT1 and SirT2 deacetylases which act to deacetylate p53 as well as other targets (*117*). SirT1 is known to deacetylate p53 at

ser382 decreasing its activity and Tenovin was also shown to increase p53 acetylation at ser382 and increase its activity (*108*).

The conformation of p53 is also important for its activity. A recent study by Fraser et al. (2010) demonstrated that the phosphorylation of p53 at either of two residues (ser215 and ser269) can promote p53 to attain an unfolded mutant-like conformation which is transcriptionally inactive; this may be an important mechanism of p53 control (*118*). This is particularly interesting in the pursuit of p53 activating therapies as inhibition of Aurora kinase which phosphorylates p53 at ser215 has been proposed as a potential chemotherapeutic. Inhibition of Aurora kinase with MLN8054 has been shown to increase p53 and p21 and leads to senescence both *in vitro* and *in vivo* (*119*). A number of Aurora kinase inhibitors are being pursued in clinical trials. The requirement for p53 in mediating the effects of Aurora kinase inhibition is unclear, it is possible that the status of p53 plays a part in determining the specific route of action but may not be specifically required for an anti-tumorigenic effect (*120*).

Factors important in attenuating p53 activity in cancer are slowly being uncovered and a number of these may well represent targets that will in the future lead to therapies. One such proposed target is TWIST, as discussed earlier the binding of TWIST to p53 prevents the phosphorylation of p53 at ser392 repressing its activity (*91*). TWIST represents a potential therapeutic target and is of particular interest as it is overexpressed in sarcoma which has relatively poor survival rates (*91*).

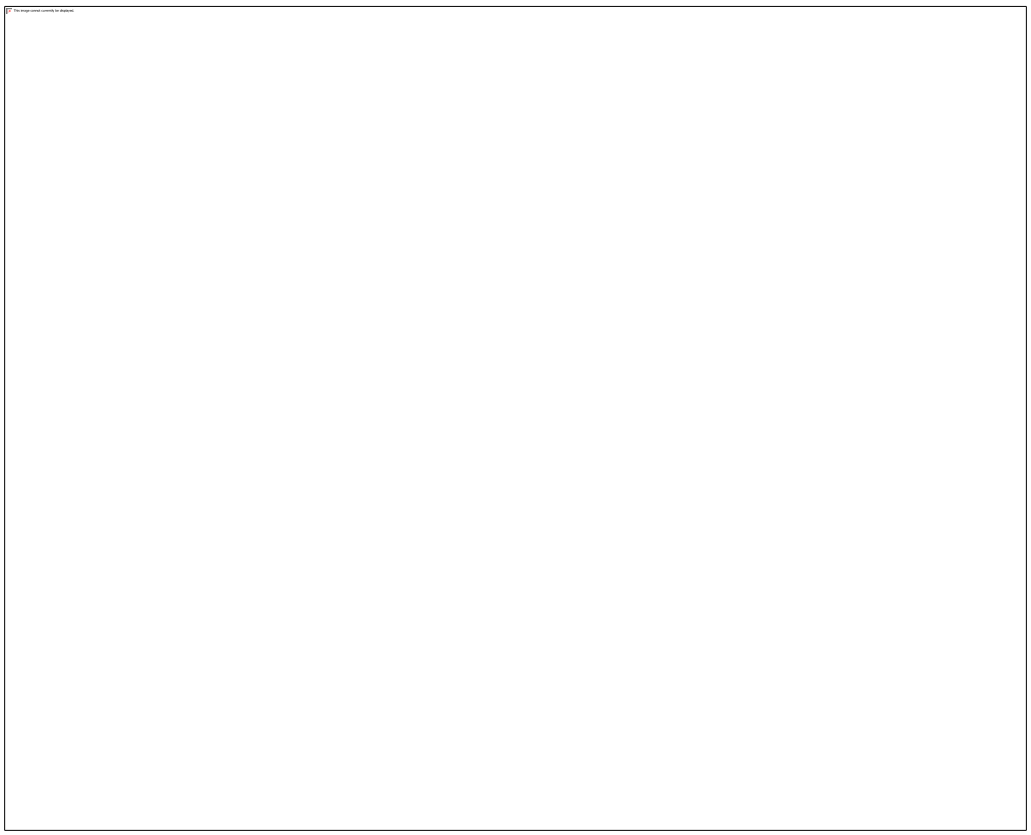

Fig 1.6 Chemical activators of wtp53.

1.10.2 Reactivation of mtp53

There are a number of potential methods by which mtp53 could be targeted (Summarized in Fig 1.4). Much effort has been put into the discovery of techniques to reactive mutant p53. Proof of concept experiments have shown that many p53 mutants are reactivatable for example it has been shown that both DNA binding and conformational mutants are rescuable by second site mutations; proving that manipulation outside of the mutation site can restore function (*121, 122*). In a further proof of concept experiment Selivanova et al. (1999) showed that short peptides derived from the C-terminus were capable of restoring some binding ability to the response element of certain p53 mutants and more critically promote p53 dependent apoptosis (*123*). The C-terminus of p53 is thought to have an allosteric negative regulatory function. The short peptides used by Selivanova et al. (1999) were shown to bind to the C-terminus and the core domain. It was suggested that it functions by displacing the C-terminus regulatory region and so helping to keep p53 in an active conformation. Additionally it may help to stabilize the core domain or promote novel contacts as the peptide did appear to be able to bring functionality to mutants with mutations in DNA contact regions.

Whilst these proof-of-concept experiments enhance our confidence that activity can be restored to certain mutants, and furthers our understanding of the control of p53, small molecules will likely have the greatest chance of yielding a clinically effective therapy. Numerous screens for molecules capable of reactivating p53 have been undertaken and have led to the discovery of a number of molecules including CP-31398, PRIMA-1, MIRA and WR-1065 (*124-127*). Whilst these all have been shown to enhance p53 binding to its response element, increase the levels of p53 controlled proteins or mediate apoptosis the mechanisms by which this happens has often proved difficult to elucidate.

CP-31398 was identified in a screen to find chemicals which could restore normal p53 DNA binding domain conformation (*126*). This was determined by binding the PAb1620 antibody which is specific to p53 with normal conformation. CP-31398 was shown to lead to p53 activation in both wtp53 and mtp53 cells and

inhibits the growth of cell lines and xenograph tumours (*126, 128*). However the mechanism is yet to be elucidated and CP-31398 does not appear to directly bind p53 (*129*). In wtp53 cells it has been shown that CP-31398 reduces p53 ubiquitination without blocking MDM2 and p53 binding (*128*). Interestingly activation of mtp53 does not lead to the transcription of all the expected activated wtp53 regulated genes and only activates a specific subset (*130*). There is however considerable debate as to whether all the effects of CP-31398 are p53 dependent or not (*131, 132*) . It appears that CP31398 has a complex mechanism of action affecting a number of pathways including p53.

Whilst PRIMA-1 was discovered in 2002 (*124*) its mechanisms of action are also still unclear. PRIMA-1 was again identified by screening and a number of reports have shown that PRIMA-1 can restore sequence specific binding and transactivation to a number of p53 mutants (*124, 133*). It has been shown that PRIMA-1 needs to be broken down in the body in order to be biologically active and one of the degradation products then alkylates thiol groups on p53 (*133*). Critically this may be a common mechanism shared with other mtp53 activators. Research has shown that oxidation reactions may be important in the regulation of p53 and that the formation of disulphide bonds inhibit its ability to bind DNA (*134*). Modifications such as the acetylation of thiol groups could prevent the formation of disulphide bonds and so it is feasible that compounds which could promote p53 reduction may be able to alter p53/mtp53 conformation and activity. The mechanism of action of PRIMA however still remains controversial and other reports have suggested that the effect of PRIMA-1 may be independent of transcription or even independent of mtp53 (*135, 136*). Such data suggests that PRIMA-1 may function via a number of complex mechanisms nonetheless PRIMA-1 appears to be a potentially useful chemotherapeutic agent and is currently in early stage clinical trials (*137*).

Another drug suggested to potentially reactivate p53 by interaction with thiol groups on p53 is MIRA (*127*). MIRA was shown to increase the proportion of mtp53 with wild-type-like conformation, increase DNA binding as well as the expression of p53 responsive genes (*127*). Furthermore it was shown to have anti-

tumourigenic effects both *in vitro* and *in vivo* however did show toxicity in mice at higher doses (*127*). It was found that a number of MIRA-1 analogs showed the ability to reactive p53 and critical to this activity was the presence of a reactive group that was surmised to be able to react with thiol and amino groups (*127*).

WR-1065 is an aminothiol with cytoprotective effects in normal cells compared to cancer cells (*138*). It has also been shown that WR-1065 can also cause the activation of wtp53 as well as mtp53 (*125, 139*). North et al. (2002) showed that WR-1065 was able to restore partial function to a temperature sensitive p53 mutant at a non-permissive temperature (*125*). This was concomitant with an increase in p53 in the normal conformation suggesting that WR-1065 is capable of modifying p53 conformation to increase its activity. Similar to PRIMA-1 and MIRA-1 this compound also contains reactive groups which may directly interact with p53 and it has been shown that MIRA-1 can cause the reduction of p53 which enhances its DNA binding ability (*139*).

In an alternative approach to identify compounds that are able to increase the thermostability of p53, Boeckler et al. (2008) used an *in silico* screening approach to identify a compound which increases the activity of p53-Y220C. This mutation causes a crevice in the structure which reduces its thermostability (*140*). The authors used a modeling approach to select drugs which would bind in this crevice. PhiKan083 was shown to bind in this crevice and enhance the stability of the protein. However further work will be required to assess the biological effect of the drug *in vitro* and *in vivo*.

Further investigations into the mechanisms of molecules that reactive p53 will help to understand p53 regulation as well as allow rational design to optimize these compounds and discover new ones.

1.10.3 Degradation of mtp53

Reactivation of mt53 represents the ideal therapeutic scenario as it would both remove the pro-oncogenic signal (mtp53) that the cancer is dependent upon and also reintroduce the tumour suppressor signal provided by a functionally

normal p53. However this represents a huge challenge and it is possible that some mtp53 is too abnormal to be refolded. An alternative and somewhat more simplistic method is to just reduce the levels of p53. To date only a small number of drugs have been identified which are able to accomplish this, which is likely due to the fact that research has mainly focussed on identification of compounds which can reactivate mtp53. Nonetheless proof-of-concept experiments have shown that siRNA knockdown of mtp53 in cells leads to cell death indicating that many tumours may be reliant on oncogenic mtp53 (*52, 141*). Furthermore introduction of mtp53 into null cells increases resistance to classic chemotherapeutics (*32*) suggesting that combined therapy may be useful.

As discussed above (see Regulation of mtp53) the Hsp90 inhibitor 17-AAG can induce the degradation of a number of oncogenic proteins including mtp53 (*51*). Inhibiting Hsp90 prevents this chaperone protein from binding mtp53 and allows it to become available to the degradation machinery (*51*). This approach is useful as it exploits a mechanism that is altered in the processing of mtp53. Because mtp53 binds much more stably to Hsp90 than wtp53 it has little effect on wild-type p53 levels (*51*). Hsp90 inhibitors are considered to be a potentially very useful therapeutic and a number of them are currently in clinical trials (*62*). Although it should be noted, that these inhibitors lead to changes in a number of oncogenic proteins not just p53.

As discussed previously (see Activation of wtp53) PRIMA-1 is a drug that was identified as capable of reactivating p53 and leading to apoptosis. Its mechanism of action however is complex and contradictory evidence exists as to how it affects mtp53. It has been suggested that its ability to cause apoptosis may be better linked to its ability to reduce mtp53 rather than activate it. Russo et al. (2010) showed that PRIMA-1 leads to the degradation of mtp53 by the proteasome (*142*). This result may not however be contradictory to previous results showing that PRIMA-1 reactivates mtp53; if mtp53 achieves a more normal conformation it may help it to become available to the degradation machinery and so levels of the highly accumulated protein would fall.

Interestingly arsenic which is used to treat acute promyelocytic leukemia has also been shown to be able to promote degradation of a number of proteins including mtp53 (*143*). Conversely arsenic does not cause the degradation of wtp53 but induces its expression (*143*). However arsenic clearly does not represent an advance in therapy and has severe toxic side effects. Nonetheless it may be useful to deduce the mechanism of action so that a more specific and less toxic therapy can be identified.

Finally, whilst p53 is normally degraded by the proteasome it may also be useful to consider other routes of degradation. Abnormally aggregated protein are often degraded by autophagy. Autophagy is often altered in cancer and it has been shown that mtp53 can inhibit autophagy (*144*). Rodriguez et al. (2012) however showed that if cells are deprived of glucose then this can reduce mtp53 acetylation and promote its degradation by autophagy (*145*). This led to a robust autophagy activation leading to cell death. They further showed that when nude mice were fed a low carbohydrate diet this reduced tumour formation by injection of cancer cells compared to controls. The authors suggest that if further work supports the generality of their results a low glucose diet may be useful for people with Li-Fraumeni syndrome. Whilst diet is clearly unlikely to cure cancer understanding such pathways clearly enhances our chances of identifying useful targets to lower p53 levels.

1.10.4 Suppression of mtp53 activity

Mtp53 leads to a number of oncogenic changes and thus research into mtp53 can lead to the identification of new targets. Targeting of such proteins may not always be reliant on p53 as such factors may be controlled by a number of mechanisms. However one drug which has specifically been identified to interfere with mtp53 activity is RETRA. RETRA was discovered in a high-through put screen of cells with a p53 activated β-gal reporter gene (*146*). RETRA was shown to activate apoptosis in cells with mtp53 but not wtp53 (*146*). This activation was also shown to be p73 dependent and functions by preventing mutant p53 repression of p73, thus

allowing p73 to activate p53/p73 target genes. The exact mechanisms by which this alleviation of repression occurs needs to be further investigated however RETRA provides an interesting alternative pathway by which p53 can be targeted in certain cancers.

1.17 Aims

As discussed above p53 is thought to be a useful potential target in the treatment of cancer and p53 could be targeted via a number of mechanisms depending on the p53 status within the cell. Investigation of the p53 pathway has revealed that a large numbers of proteins are important in the regulation of p53 stability, localisation and activity. As discussed a number of p53 activators have been identified and are in various stages of development. However the identification of other such targets will provide us with more potential tools to attack different cancers and help further our understanding of p53 in cancer. Additionally by comparison very little is known about the regulation of mtp53. Clearly investigation into the control of mtp53 is vital as mtp53 is present in around half of all cancers and can be critical to tumour survival.

This project initially aimed to investigate two of the potential ways of targeting p53, namely activation of p53 in cells with wild type p53 and degradation of p53 in cells with mutant p53. This was accomplished using various screening methods to identify compounds capable of these effects (Chapter 3). As will be described screening led to the identification of a number of inhibitors able to affect either the activity or stability of mtp53 or wtp53 respectively. Of particular interest were two IKKβ inhibitors which lead to the reduction of mtp53. Thus the focus of this work was then to further characterise inhibition of this pathway in terms of its effect on p53. The aim was to use classic molecular and biochemical techniques (Chapter 4) as well as proteomic techniques (Chapter 5) to elucidate important effector and co-regulated proteins within this pathway. The ultimate goal was to better understand factors important in stability of p53 and p53 pathway proteins.

Chapter 2

Materials & Methods

2. Materials and Methods

2.1 Reagents

All Reagents were purchased from Sigma Aldrich unless otherwise stated. All tissue culture reagents including DMEM, RPMI and Trypsin were purchased from Invitrogen. FBS was purchased from Biosera.

2.2 Equipment

DNA concentration was measured using a NanoDrop spectrophotometer. Bradford and BSA assay was measured on a PerkinElmer VICTOR 1420 Multilabel counter. Alamar Blue intensity was measured on a PerkinElmer Envision Multilabel reader. qPCR was conducted using a PTC-200 Peltier Thermal cycler. PCR was conducted using a PTC-225 Peltier Thermal cycler. X-ray films were developed using a Mediphot 937 developer. SDS-PAGE was carried out using Biorad II Minigel System. In cell western results were analysed using an Odyssey SA plate reader. Mass spectrometry analysis was completed using an ABSciex Qstar Elite or ABSciex 5600.

2.3 Cell procedures:

2.3.1 Tissue culture

All cells were grown in humidified incubators at 37^{o}C. The CO_2 saturation for each cell line is indicated in Table 2.1. All cells lines were grown in either RPMI or DMEM as indicted in Table 2.1. Cells were passaged between 1-3 times per week as required. Passaging was conducted as follows. Media was discarded and then cells were washed with sterile PBS (10ml/10cm plate) and Trypsin was added (2ml/10cm Plate). Plates were then incubated for ~5-10mins at 37^{o}C until cells became detached from the plate. Media was then added to plate (8ml/10cm plate) and cells were then re-plated at the desired dilution.

Table 2.1 Cell lines and culture conditions used in this study

Name	Cancer Type	p53 Status	Media	% CO_2	Maximum Dilution at passage
T47D	Breast	L194F	RPMI	5%	1:5
A375	Melanoma	Wild type	DMEM	10%	1:10
ARN8	A375 with stabily integrated p53-βgal reporter gene	Wild type	DMEM	10%	1:10
BT-549	Breast	R249S	DMEM	5%	1:5
H1299	non-small cell lung carcinoma	Null	RPMI	5%	1:10
MDA-MB-231	Breast	R280K	RPMI	5%	1:10

2.3.2 Freezing of cells

For long-term storage all cells were kept in liquid nitrogen storage tanks. Cells were grown to confluency in a 10cm plate. Cells were then trypsinised by the same procedure as during cell passaging. Once the cells had detached, and media was added, cells were pelleted by centrifugation at 1000rpm for 5mins. The media and trypsin was then discarded. Cells were then resuspended in 5mls of cell freezing solution composed of 10% DMSO in FBS. The resuspended cells were then split into ~5 Nunc cryovials. Vials were then frozen at a rate of 1^{o}C per minute in an isopropranol bath before being transferred to liquid nitrogen storage. When cells were recovered they were rapidly thawed in a 37^{o}C water bath before being plated into 10cm plates with 9ml of media. After 24Hrs the media and freezing solution was discarded and fresh media added. Cells were then grown to confluency before their first passage.

2.3.3 Drug treatment

All drugs were suspended in DMSO and stored in aliquots according to manufactures recommendations.

Cells were seeded in 10cm, 6cm, 6well, 24well, or 96well plates 24Hrs prior to treatment. The compounds were diluted to the appropriate concentration (Detailed in Table 2.2) in cell culture media. As a control DMSO only was also diluted to the equivalent concentration to media. These were then transferred to the cells and cells incubated for the stated time.

Table 2.2 Compounds used in this study

Drug	Concentration	Supplier
Tocris kinase inhibitor toolbox (for details see supplementary data)	50µM-0.4 µM	Tocris
LOPAC compounds (for details see chapter 1)	50µM-0.4 µM	Sigma
MG132	10 µM	Sigma
Cycloheximide	30 µg/ml	Sigma
17-AAG	5uM-20nM	LC Laboratories
Nutlin	20uM-160nM	Enzo Life Sciences
LMB	30uM-230nM	Sigma
Roscovitine	50µM-0.4 µM	Sigma
BMS-345541	50µM-0.4 µM	Sigma
DMSO control	Equivalent quantity to test compound	Sigma

2.3.4 Plasmid Transfection

Transfection mixture (10cm Plate)

5-10µg Plasmid DNA

10-15µl Attractene

Made up to final volume of 300µl with serum free media

Cells were seeded 24Hrs prior to transfection and grown to 40% confluency. Media was discarded and fresh added just prior to transfection. Transfection mixture was then added drop wise to the cells.

2.3.6 FRT Stable cell selection

Cells were transfected with the required plasmid and pOGG44 plasmid at a ratio of 1:9 (0.5µg + 4.5µg or 1µg + 9µg respectively). A control plate of cells was transfected with pOGG44 only. 24Hrs after transfection media was supplemented with hygromycin to a final concentration of 50mg/ml. Media and hygromycin was refreshed every 2-3 days for ~2weeks until control cells had died and colonies were evident on the other plates. Colonies was then trypsinised from the plates, seeded in 10cm plates and grown to confluency.

2.3.7 siRNA treatment

Transfection solution 1

190µl Serum free media

4µl siRNA (from 20µM stock)

Transfection solution 2

190µl Serum free media

4µl Dhamafect 4

Cells were seeded in 6 well plates 24hrs prior to treatment. Media was discarded and fresh added just prior to transfection. Transfection solutions were made up as indicated and incubated for 5 mins at room temperature before the two solutions were combined. This solution was then incubated for a further 20 mins at room temperature before being added drop-wise to cells. If required a second transfection of siRNA and control was conducted 24Hrs later (see Table 2.3).

Table 2.3 siRNA used in this study

Target	Final concentration	Number of siRNA transfections
IKKβ	40nM	2
MDM2	40nM	1

MS hits library	40nM	1

2.3.7 Harvesting cells

When harvesting all plates were kept on ice. Media was discarded before cells were washed with cold phosphate buffered saline (PBS). Cells were scraped into 1ml cold PBS using a cell scraper and transferred to an eppendorf. Cells were then centrifuged at 5000rpm for 5Mins at 4^{o}C. PBS was then discarded and pellets snap frozen in liquid nitrogen.

2.3.8 Cell lysis

NP40 Lysis Buffer

1% NP40

150mM NaCl

50mM Tris-HCl pH8

1mM DTT

+ Protease inhibitor cocktail (Roche)

Cells were lysed in NP40 lysis buffer unless otherwise stated. Cell pellets were suspended in 3 times pellet volume and incubated on ice for 20mins. The solution was then centrifuged at full speed for 10mins at 4^{o}C in order to remove any insoluble material. Supernatant was then collected and either snap-frozen in liquid nitrogen and stored at -80^{o}C or immediately used for further analysis.

2.4 Assays:

2.4.1 Determination of protein concentration

For general use including protein concentration determination prior to SDS-PAGE protein concentration was determined by Bradford assay. For protect concentration determination prior to mass spectrometry BCA assay was used.

2.4.2 Bradford assay

Bradford reagent

0.01% (w/v) coomassie blue G250

4.25% Ethanol (v/v)

8.5% Phosphoric acid (v/v)

BSA standards (0.25-4mg/ml) were diluted 1µl in 200µl of Bradford reagent in a 96 well plate (in triplicate). The same volume of each sample of unknown concentration was also added to 200µl Bradford reagent (in triplicate). The absorbance was then read at 595nm using a VICTOR 1420 multilabel plate reader. A standard curve was then created using absorbances for the BSA standards. Sample concentrations were then calculated from standard fit.

2.4.3 BCA assay

BCA assay was conducted using the Pierce BCA assay kit. Working reagent was prepared by combining reagents A and B at a ratio of 50:1. 10µl BSA standards/samples were then combined with 200µl working reagent in 96 well plates. Plates were then incubated at 37^{o}C for 30mins. The absorbance was measured at 562nm. As with the Bradford assay, read-outs were converted to concentration with the use of a standard curve created from BSA standards.

2.4.4 β-gal Assay

Fixing solution

2%formaldehyde

0.2% Glutaldehyde

ARN8 (containing a stability integrated p53 β-gal reporter gene) cells were grown in 24 well plates and treated as indicated. The media was then discarded and cells were washed with 1ml PBS. The cells were then incubated with 300µl fixing solution

for 10mins at room temperature. Cells were then washed twice in 1ml PBS. 300µl of staining solution was then added to each well and incubated at 37^{o}C for 2Hrs. Cells were then visually inspected for the blue colouration.

2.5 Microbiological techniques:

2.5.3 Bacterial culture on agar plates

LB agar (Luria-Bertani)

1 % (w/v) Tryptone

1 % (w/v) NaCl

0.5 % (w/v) Yeast extract

1.5 % (w/v) Agar

Antibiotics: Ampicillin (50µg/ml), Kanamycin (100µg/ml)

LB agar was melted in a microwave and allowed to cool to approximately body temperature to the touch. If antibiotics were required they were added at the appropriate concentration. The agar was then poured into 10cm petri dishes and left to set. Plates were stored at 4^{o}C. Bacterial cultures were inoculated onto the plates either by picking colonies from previous plates or glycerol stocks and streaked using a sterile loop or by plating directly from transformed cultures.

2.5.4 Bacterial culture in broth

LB Broth (Luria-Bertani)

1 % (w/v) Tryptone

1 % (w/v) NaCl

0.5 % (w/v) Yeast extract

Antibiotics: Ampicillin (50µg/ml), Kanamycin (100µg/ml)

5ml LB Broth (supplemented with selective antibiotics if required) was inoculated with a bacterial colony. This was incubated at 37^{o}C for ~6Hrs at 225rpm. This starter culture was then added to 250mls LB (again supplemented with selective antibiotics if required) and incubated in the same conditions overnight.

2.5.1 Preparation of competent cells

Buffer 1

30mM Potassium acetate

100mM $RbCl_2$

10mM $CaCl_2$

50mM $MnCl_2$

15% (v/v) Glycerol

Adjusted to pH 5.8 with acetic acid

Sterilised by filtration

Buffer 2

10mM MOPS

75mM $CaCl_2$

10mM $RbCl_2$

15% (v/v) Glycerol

Adjusted to pH 6.5 with KOH

Sterilised by filtration

A 5ml LB Broth was inoculated with a DH5α bacterial colony from a stock plate. This was incubated at 37^{o}C overnight at 225rpm. This starter culture was then added to 250mls LB and incubated in the same conditions until the OD_{600nm} was between 0.3-0.5. The cells were pelleted by centrifugation (10min, 8000rpm, 4^{o}C) and then resuspended in 80ml cold buffer 1. The sample was then incubated for 10mins on ice. Cells were again pelleted (5mins, 2500rpm, 4^{o}C) and resuspended in 8mls cold buffer 2. Cells were incubated on ice for 15mins and aliquoted into 50µls. Samples were snap-frozen in liquid nitrogen and stored at -80^{o}C.

2.5.2 Transformation of bacteria

100µl of DH5α cells were defrosted on ice and 0.1-0.5µg of plasmid DNA was then added to the cells. The cells were incubated on ice for 1hr. The cells were then heat-shocked by incubation in a water bath for 2 min at 42°C. The cells were then incubated on ice for a further 2 mins prior to the addition of 1ml LB media. The culture was incubated at 37 °C at 225 rpm for 30 mins. The culture was subsequently plated onto LB agar plates overnight at 37 °C.

2.5.5 Glycerol stocks

Glycerol stocks were used for the long-term storage of bacteria. 200µl of overnight bacterial culture in LB broth was added to 800µl of Glycerol. This was then snap-frozen and stored at -80^{o}C.

2.5.6 Maxi/Mini preps

Maxi/mini preps were used for purification of plasmid DNA from bacteria. DH5α cells were transformed with the appropriate plasmid. The bacterial cells were grown up in LB broth as described above. The bacterial cells were then pelleted by centrifugation at 6000rpm for 20mins at 4^{o}C. DNA was isolated using Qiagen Mini or Maxi prep kits according to manufacturer's instructions. DNA was eluted in nuclease-free H_2O and stored at -20^{o}C. DNA concentrations were determined using a Nanodrop spectrophotometer.

2. 6 Molecular Biology techniques:

2.6.1 SDS-PAGE and Western blotting

<u>10% Resolution gel:</u>

30 % Acrylamide mix (30 % (w/v) acrylamide and 0.8 % (w/v) bis-acrylamide)

1.5 M Tris (pH 8.8)

10 % SDS

10 % Ammonium persulphate

TEMED

Stacking gel:

30 % Acrylamide mix (30 % (w/v) acrylamide and 0.8 % (w/v) bis-acrylamide.)

1.5 M Tris (pH 6.8)

10 % SDS

10 % Ammonium persulphate

TEMED

SDS sample buffer

125 mM Tris pH 6.8

5 % (w/v) SDS

25 % (v/v) Glycerol

0.02 % (w/v) Bromophenol blue

DTT (1 M) added fresh to buffer prior to use

Running Buffer:

192 mM Glycine

25 mM Tris

0.1 % (w/v) SDS

Transfer Buffer:

192 mM Glycine

25 mM Tris

20 % (v/v) Methanol

Gels were made using Biorad system. Approximately 4ml of resolution buffer was made fresh and poured between the glass plates. Water was then added to the top to ensure a clean, straight edge to the resolution gel. The gel was then left to polymerise. Once set the water was poured off, stacking buffer was added and a comb was added to create the well. The stacking buffer was then left to polymerise. 20µg of sample was combined with sample buffer. This was then boiled at 95^{o}C for 5mins. A protein ladder and the samples were then loaded onto the gels in biorad tanks. Running buffer was then added to the tanks and the gels run at 180-200V for ~1hr until the bromophenol blue front reached the end of the gel. The glass plates were then opened up and the stacking gel sliced off. The gels were then gently transferred to blotting paper. Nitrocelulose membrane was added to the top of the gel and a second piece of blotting paper was added to the top. This was then sandwiched between 2 pads under transfer buffer to remove bubbles. This was then encased in a biorad cassette. The gels are then transferred in the biorad tanks at 300mA for 90mins or 30mA overnight.

2.6.2 Immunoblotting

ECL Solution 1

100 mM Tris pH 8.5

2.5 mM Luminol

0.4 mM p-Coumaric acid

ECL Solution 2

100 mM Tris pH 8.5

0.02 % (v/v) H2O2

Solutions stored at 4 ºC and protected from light

5% Milk

5% Dry low fat milk powder rehydrated in PBS-t.

Following SDS-PAGE and transfer, membranes were blocked in 5% milk for 1hr at room temperature with shaking. The membrane was then incubated with primary antibody diluted to the appropriate concentration (as shown in Table 2.4) either at room temperature for 1hr or at 4^{o}C overnight. The blot was then washed 3 times for 5 mins each with PBS-t (0.1% (v/v) Tween 20 in PBS) to remove unbound antibody. The blot was then incubated with the appropriate secondary antibody (see Table 2.4) at room temperature for 1hr. Again the blot was then washed 3 times for 5 mins each with PBS-t to remove excess antibody. The membrane was then incubated with ECL1 and ECL2 solutions mixed at a 1:1 ratio for 1min at room temperature. X-ray film was then overlaid on the membranes and then developed to visualise the protein bands.

Table 2.4 Antibodies used in this study

Antibody name	Supplier	Secondary antibody (Western blot)	Secondary antibody (In cell western)	Dilution (western blot)	Dilution (In cell western)
DO1	Gifted by Borek Vojtesek (Masaryk Memorial Cancer Institute)	Rabbit anti mouse	Donkey anti mouse	1:10000	1:2000
2A10	Gifted by Borek Vojtesek (Masaryk Memorial Cancer Institute)	Rabbit anti mouse	Donkey anti mouse	1:2000	1:800
4B2	Gifted by Borek Vojtesek (Masaryk Memorial Cancer Institute)	Rabbit anti mouse	Donkey anti mouse	1:1000	1:1000
IKKβ	Abcam	Sheep anti rabbit	-	1:1000	-
IKBα	Abcam	Swine anti rabbit	-	1:1000	-
IKBα-P	New England Biolabs	Swine anti rabbit	-	1:1000	-
p21	Calbiochem	Rabbit anti mouse	-	1:1000	-
β-Actin	Abcam	Rabbit anti mouse	-	1:5000	-
RPS3	ProteinTech group	Rabbit anti mouse	-	1:1000	-
Cyclin D1	Calbiochem	Rabbit anti mouse	-	1:1000	-
Cyclin D3	Santa Cruz	Rabbit anti mouse	-	1:1000	-

2.6.3 In cell western

PBS-Triton

1% (v/v) Triton in PBS

Fixative solution

3.8% (v/v) Formaldehyde in PBS-t

3% BSA

3% (w/v) Bovine serum albumin in PBS-t

Cells were seeded in 96 well plates 24Hrs prior to treatment. Cells were then treated with the appropriate compound for the indicated time. After appropriate incubation the media is discarded and fixative solution added (100µl/well). The plates were then incubated at room temperature for 20mins. The cells are then permeabilised by 5 x 5min washes with PBS-Triton. The wells are then blocked to prevent non-specific antibody binding with 3% BSA for 1Hr30mins at room temperature with shaking. All wells with the exception of 1 control well per plate were then incubated in primary antibody diluted (As indicated in Table 2.4) in 3% BSA for 2Hrs at room temperature with shaking or overnight with shaking. The wells are then washed 5 times in PBS-t for 5mins each. Secondary antibody (diluted to 1:1000) and DRAQ5 (diluted to 1:10000) in 3% BSA was then added to each well. The control well was incubated with secondary antibody only. Plates were incubated with shaking for 1hr at room temperature and protected from light. Wells were then washed 5 times with PBS-t for 5mins each. Plates were then scanned using an Odyssey SA plate reader.

For all analyses average intensities were used. All antibody fluorescence levels were normalised against the secondary only control by deducting secondary only value from the primary values. These values were then normalised to total cells number by dividing by DRAQ5 intensity.

2.6.4 DNA-sequencing

DNA sequencing was carried out using the Big Dye Terminator V3.1 cycle sequence kit as follows.

Master Mix	Parameters
1µl Template	96°C 1 min
10µM Primer (as indicated in Table 2.5)	96°C 10 secs
2µl Big Dye	50°C 5 secs
2µl Big dye sequencing buffer	60°C 4 mins
Up to 10µl with H_2O	Cycle to step 2 x30

The reaction mix was made up as indicated. The reaction was then conducted on a thermocycler at the indicated parameters.

EDTA/Ethanol precipitation

The PCR product was then transferred to a new tube and 2.5µl of 125mM EDTA added. 30µl of 100% Ethanol was then added to each sample to precipitate the DNA. Samples were mixed by vortexing and then incubated at room temperature for 15mins. The precipitated DNA was then pelleted by centrifugation (13000rpm, 20mins) and the ethanol was removed. Samples were then centrifuged for a further 5mins and any remaining ethanol removed. 30µl of 70% Ethanol was then added to each sample and samples were centrifuged at 13000rpm for 5mins at 4°C and the Ethanol discarded. Samples were centrifuged again at 13000rpm for 5mins at 4°C and any residual ethanol removed. Samples were then allowed to dry in a fume hood until no liquid remained. The sequences were then analysed by SourceBioscience (Cambridge).

Table 2.5 Sequencing primers used in this study

Name	Site	Sequence
IKKβ-pEF5/FRT/V5-DEST 1	Within pEF5/FRT/V5-DEST 1 vector prior to gene integration site	GTAAAACGACGGCCAG
IKKβ-pEF5/FRT/V5-DEST 2	Within IKKβ	CAAGCTCTTTACCCTACCC
IKKβ-pEF5/FRT/V5-DEST 3	Within IKKβ	GCCACACATTGGACATGGA
IKKβ-pEF5/FRT/V5-DEST 4	Within IKKβ	CTGCTGCTGGCCTGGAG

2.6.5 qPCR

2.6.5.1 RNA extraction

Cells were seeded in 6 well plates and treated as indicated. After treatment cells were harvested as normal and RNA was extracted using a Qiagen RNeasy kit in accordance with manufacturer's instructions. Samples were homogenised using QIAshredder columns. RNA was eluted using RNase free water and immediately used to synthesise cDNA as detailed below

2.6.5.2 Synthesis of cDNA from RNA

<u>RT reaction mixture</u>

2µl 10x RT Buffer

2µl 5µM dNTP

2µl 10x RT Buffer

0.2µl 0.5mg/ml oligo dT primes

0.2µl 40 U/µl RNase inhibitor

2µl 100mM DTT

5.6µl nuclease free water

0.5µg RNA was diluted in 7µl of water and incubated at 65^{o}C for 5 minutes. RNA was cooled and combines with 13µl RT reaction mixture. The mixture was then incubated for 1hr at 37^{o}C. cDNA was then used for qPCR.

2.6.5.3 qPCR reaction

Reaction mix	Cycle parameters
12.5µl Solaris master mix	95^{o}C 15 mins
Solaris primer/probe set	95 ^{o}C 15 secs
250-1000ng cDNA	60 ^{o}C 60 secs
Made up to 25µl with H_2O	Cycle to step 2 40 times

The reaction mixture was set up as indicated in white qPCR tubes with clear optical lids. The reaction was run on a PTC-200 Peltier Thermal cycler using the indicated cycle parameters. When the Ct values were obtained relative change was calculated using the equation $2^{-\Delta\Delta Ct}$. ΔCt indicates the difference between average ct for the gene of interest and average ct for control gene (e.g. β-actin). ΔΔCt indicates the difference between ΔCt for the treatment condition and control condition (e.g. DMSO).

2.6.6 Gateway cloning

In order to clone the gene of interest (GOI) the following Gateway system (Invirtogen) protocol was used. Firstly the GOI (IKKβ) was flanked by attB sites was generated (2.4.8.1). Next this was inserted into the entry vector using the BP reaction (2.4.8.2). Finally the gene was transferred to the destination clone (2.4.8.2).

2.6.8.1 Producing IKKβ-AttB product

Master mix	Cycling Conditions
5µl 10 x PFU master mix	Activation: 95^{o}C 15mins
1µg Template (IKKβ- pCMV2)	Denaturation: 95^{o}C 30Secs
0.1µM Forward and Reverse primers	Annealing: 56 ^{o}C 30secs
Up to 50µl with ddH_2O	Elongation: 72^{o}C 1min
	Cycle to step 2 30 times
	72^{o}C 5mins
	4^{o}C ∞

PCR was used to amplify the IKKβ gene from an IKKβ- pCMV2 vector (obtained from addgene). The primers designed (Detailed in Table 2.6) also contained leader sequences with Att sites in order to obtain a product of IKKβ flanked by Att sites. PCR master mix was made as indicated and the reaction was then run at the indicated cycling conditions in thin walled PCR tubes.

Table 2.6 PCR primers used in this study

Name	Site	Sequence
IKKβ Forward (With leader Att sites)	IKKβ start	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCG AAGGAGATAGAACCATGAGCTGGTCACCTTCC
IKKβ Reverse (With leader Att sites)	IKKβ End	GGGGACCACTTTGTACAAGAAAGCTGGGTCCT ATCATGAGGCCTGCTCCAG

2.6.10 BP reaction

Reaction mixture

15-150ng att-PCR product

150µg pDONR221

2µl BP Clonase

Up to 8µl TE Buffer pH8

The IKKβ PCR product (flanked with Att sites) was incorporated into the pDONR221 vector using a BP reaction. The reaction mixture was made up as indicated and incubated at room temperature overnight. The reaction was then stopped using 1µl proteinase K (37^{o}C, 10mins). 50µl of DH5α were then transformed using 1µl of the reaction sample. The transformed bacteria were then plated onto LB agar plates supplemented with KAN and incubated at 37^{o}C overnight. Individual colonies were then picked and grown up in 5ml LB broth. The plasmid DNA was then isolated using the miniprep kit. Plasmids were then sequenced to confirm the BP reaction was successful.

2.6.11 LR Reaction

Reaction mixture

150ng entry clone

150ng Destination vector

2µl LR Clonase

Up to 8µl with TE buffer

An LR reaction was used to transfer the IKKβ gene from the entry clone (pDONR221) to destination vector (pEF5/FRT/V5-DEST 1). The reaction mixture was set up as shown and incubated overnight at 25^{o}C. To stop the reaction 1µl of proteinase K was added and the sample heated to 37^{o}C for 10mins. 50µl of DH5α were then transformed using 1µl of the reaction sample. The transformed bacteria were then plated onto LB agar plates supplemented with AMP and incubated at 37^{o}C overnight. Individual colonies were then picked and grown up in 5ml LB broth. The plasmid DNA was then isolated using the miniprep kit. Plasmids were then sequenced to confirm the LR reaction was successful.

2.6.7 Site-directed mutagenesis

Master mix	Cycle parameters
5µl 10 x PFU master mix	95^{o}C 15 mins
50ng Template	95^{o}C 30 Secs
0.125µM Forward and Reverse primers	55^{o}C 30 Secs
Up to 50µl with ddH_2O	72^{o}C 1 mins
	Cycle to step 2 x30

Specific mutations in the IKKβ gene (within the pEF5/FRT/V5-DEST 1 vector) were created using site-directed mutagenesis. The reaction mixture was made up as shown and the reaction conducted on a thermocycler using the indicated cycle parameters. 10µl of the PCR product was transferred to a new tube (no dpn control). 1µl of dpn was then added to the remaining 40µls and incubated for 3Hrs at 37^{o}C. 50µl DH5α were then transformed with 1µl of the reaction mixture/no dpn

control. DH5α cells were then plated onto LB agar and grown overnight at 37^{o}C. Colonies were picked and grown up in 5ml LB broth overnight. Plasmid DNA was then extracted by mini-prep and sequenced.

Table 2.7 Mutagenesis primers used in this study

Name	Sequence
IKKβ K44M Forward	CAGATTGCCATCATGCAGTGCCG
IKKβ K44M Reverse	CTGCCGGCACTGCATGATGGCAA
IKKβ S177E/S181E Forward	GATCAGGGCGAACTTTGCACAGAATTCGTGGGG
IKKβ S177E/S181E Reverse	CCCCACGAATTCTGTGCAAAGTTCGCCCTGATC

2.7 Mass spectrometry

All Mass-spectrometry work was completed in Prof. Mark Molloy's Lab/APAF (Australian Proteomics Analysis Facility) in Sydney, Australia. All sample preparations and MS analysis were completed with the help of Thiri Zaw (APAF). All SCX work was completed by Natasha Lucas (APAF). Data analysis work was completed with the help of Thiri Zaw, Natasha Lucas, Robert Parker and Mark Molloy (APAF and Molloy group).

2.7.1 iTRAQ proteomics experiment:

2.7.1.1. iTRAQ sample preparation: Cell treatment, lysis and trypsinisation

Lysis buffer

0.15%SDS

20mM HEPES pH7.5

150mM NaCl

1mM EDTA

1mM EGTA

+Protease inhibitors

T47D cells were seeded in 20cm tissue culture plates to reach 80-90% confluency 24hrs later. They were then treated with 5µM IMD0354. Plates were harvested 0, 30, 60 and 120mins after treatment. Prior to harvesting, cells were washed 3 times in cold PBS to ensure all media and FBS was removed. Cells were centrifuged for 3mins at 3000rpm and the supernatant discarded. Cells were then resuspended with 1ml SDS lysis buffer. They were briefly sonicated and then incubated on ice for 20mins. Cells were then centrifuged (13500rpm 10mins) to remove insoluble fragments; the supernatant was kept for analysis. Protein concentration was determined by BCA assay. 100µg of each sample was then diluted 1:3 with ddH_2O.

2.7.1.2. iTRAQ sample preparation: Sample reduction, alkylation and trypsinisation

TCEP was added to each of the 100µg lysate samples to give a final concentration of 5mM and incubated at 60^{o}C for 1hour with shaking. MMTS was then added to give a final concentration of 10mM. Samples were incubated at room temperature for 10mins. 4µg of Trypsin was then added to each sample and samples were incubated at 37^{o}C overnight. Samples were lyophilized using a vacuum centrifuge for ~1-3Hrs and pellets were resuspended in 30ul TEAB.

2.7.1.3. iTRAQ sample preparation: Labelling reaction

iTRAQ labels (114, 115, 116, and 117) were resuspended in 70ul Ethanol and added to the prepared samples. Samples were incubated at room-temperature with shaking for 2hrs. The samples were then checked to ensure that they had been successfully labelled. To do this 2µl of each sample was then combined and de-salted by Zip-tipping (Millipore). Zip-tips were activated by washing 5 times with 5µl

of acetonitrile, 0.1% TFA and then washed 5 times with 5µl 0.1% TFA. 5µl of the sample was pipetted and repipetted a total of 10 times. Zip tips were again washed 5 times with 0.1% TFA. De-salted sample was then eluted with 2µl 4mg/ml MALDI matrix resuspended in 70% acetonitrile, 0.06% TFA and spotted onto a 384 Opti-TOF matrix. Additionally MALDI peptide standards were also pipetted onto the plate. Samples were then analysed on an ABSciex 4800 Plus Proteomics analyser automated MALDI-TOF/TOF to check the samples had been labelled. Once this was established all samples were then combined and lyophilised by vacuum centrifugation for 3-4Hrs. Samples were then subject to Strong cation exchange.

2.7.1.4. iTRAQ sample preparation: Strong cation exchange

Strong cation exchange was completed by the Australian Proteomics Analysis Facility (APAF). Once the fractions had been separated they were resuspended in 100µl 0.1%TFA, 2% acetonitrile. Fractions with lower peptide content were pooled to create a total of 20 separate samples for MS analysis.

2.7.1.5. iTRAQ MS/MS analysis

Samples were analysed by LC-MS/MS using an ABSciex QStar Elite mass-spectrometer and a ABSciex 5600 mass-spectrometer. Proteins were identified using ProteinPilot (Applied Biosystems).

2.7.2 SILAC phosphoproteomics experiment

2.7.2.1. SILAC sample preparation: Labelling

T47D cells were grown in SILAC control DMEM (R0K0 – “Light”) or SILAC DMEM media containing 13C and 15N labelled arginine, and 13C and 15N labelled lysine (R10K8 – “Heavy”) (Dundee cell products) for 6 passages.

2.7.2.2. SILAC sample preparation: Cell treatment and lysis

Urea lysis buffer

50mM Tris-HCl pH7.5

150mM NaCl

0.5% Triton

8M Urea

+Protease and phosphatase inhibitors

Cells were then grown in 20cm tissue culture dishes until ~80% confluent. "Heavy" labelled cells were treated with 5µM IMD0354 for 2Hrs, "Light" labelled cells were treated with DMSO for 2Hrs. Cells were then washed 5 times in cold PBS to ensure all media and FBS was removed and then scraped into 1ml cold PBS. Cells were pelleted by centrifugation (3000rpm 3mins) and the supernatant discarded. Cells were resuspended in urea buffer, they were briefly sonicated and incubated on ice for 20mins. Samples were centrifuged at top speed for 10mins. The supernatant was retained and the insoluble fraction discarded.

2.7.2.3. SILAC sample preparation: Sample reduction and alkylation

10µl 200mM DTT was added to each lysate sample and samples were then incubated for 40mins at 56^{o}C. Samples were allowed to cool to room temp and 10µl 400mM IAA was added. Samples were incubated for 20mins at room temperature in the dark. The prepared samples were then transferred to 15ml tubes for acetone precipitation.

2.7.2.4. SILAC sample preparation: Acetone precipitation

5-10 volumes of cold acetone was added to each sample. Samples were incubated at -20^{o}C overnight. Samples were centrifuged at 5000-10000g for 20mins. Acetone was removed by decanting. Any residual acetone was removed by drying in a fume hood for ~5mins.

200µl of 100mM ammonium bicarbonate/8M urea was added to samples and incubated for 30mins at room temperature to dissolve the pellet. 800µl 100mM ammonium bicarbonate was then added to reduce the urea concentration to 1.6M. Samples were then transferred to 1.5ml eppendorfs.

2.7.2.5. SILAC sample preparation: Trypsin digestion

Protein concentration was determined by BCA assay. 1mg of each sample was digested. 20µg of trypsin (Promega) was added to each sample and incubated at 37^{o}C overnight.

2.7.2.6. SILAC sample preparation: C18 Clean up

Trypsinised samples were then purified on C18 columns (Nest Group Macro spin columns). To activate columns 500µl Methanol was added to each C18 spin column. Columns were centrifuged at 100rcf for 2mins and flow-through discarded. Columns were washed with 500µl H_2O. Columns were centrifuged at 100rcf for 2mins and flow-through discarded. Samples were added to the columns (30-300µg/column). Columns were centrifuged at 100rcf for 2mins and flow though was reapplied to columns. Columns were centrifuged again at 100rcf for 2mins and flow-through discarded. Columns were again washed using 250µl H_2O which was added to each column. Columns were centrifuged at 100rcf for 2mins and flow-through discarded. This wash step was repeated twice more. Proteins were then eluted from the column with 90%ACN, 0.1%Formic acid and centrifuged at 100rcf for 2mins. Samples were then lyophilised using a vacuum centrifuge until volume was ~20µl. Samples were then subject to phosphopeptide enrichment.

2.7.2.7. SILAC sample preparation: phosphopeptide enrichment.

Buffers:

Buffer A: 80% ACN, 0.4% TFA

Buffer B: 1ml Buffer A+400µl Lactic acid

Elution 1: 20µl ammonium hydroxide + 380µl H2O

Elution 2: 20µl Pyrrolidine +380µl H_2O

2.5% TFA

The columns were prepared by washing with 20µl Buffer A which was added to each TiO_2 tip and centrifuged at 3000 x g for 2mins. Flow-through was then discarded. The tips were then washed with 20µl Buffer B and centrifuged at 3000 x g for 2mins. Flow-through was then discarded. Peptide samples were resuspended in 150µl buffer B and applied to the spin tip. Tips were centrifuged at 1000 x g for 10mins. Samples were reapplied to the spin tip and centrifuged at 1000 x g for 10mins and flow-through was discarded. The columns were washed by adding 20µl buffer B and centrifuged at 3000 x g for 2mins and flow-through was discarded. The columns were washed by adding 20µl buffer A and centrifuged at 3000 x g for 2mins and flow-through was discarded. This wash with buffer A was repeated twice more. The tips were placed in new tubes and 50µl of elution buffer 1 was added and tips were centrifuged at 1000 x g for 5mins. Using the same collection tubes 50µl of elution buffer 2 was added to each spin tip and tips were centrifuged at 1000 x g for 5mins. The eluate was then retained and acidified by adding 100µl 2.5%TFA until sample was pH2-2.5.

2.7.2.8. SILAC sample preparation: Graphite clean-up

After Phosphopeptide enrichment samples were purified using Pierce Graphite spin columns in accordance with recommendations of the enrichment kit. Columns were placed into 1.5mL collection tubes and centrifuged at 2000 × g for 1 min to remove storage buffer. 100µL of 1M NH_4OH was added to each tube and then centrifuged at

2000 × g 1 min; flow-through was discarded. This wash step was then repeated. The graphite columns were then activated by adding 100µL of acetonitrile. Columns were then centrifuged at 2000 × g for 1 min and flow-through discarded. Next 100µL of 1% TFA was added to each and centrifuged at 2000 × g for 1 min. Again flow-through was discarded. This was then repeated once. Columns were then placed into a new collection tube and the sample was applied on top of the resin bed and allowed to bind for 10 minutes with periodic mixing. The columns were then centrifuged at 1000 x g for 3 mins and the flow-through discarded. The columns were then placed into a new collection tube and the columns were washed by adding 200µL of 1.0% TFA and centrifuged at 2000 × g for 1 min; the flow-through was discarded. This wash step was then repeated. Columns were then placed into new collection tube and 100µL of 0.1% formic acid in 50% acetonitrile was added to elute the sample. Columns were centrifuged at 2000 × g for 1 min. This step was repeated three more times using the same collection tube for a total elution volume of 400µL. Samples were then dried in a vacuum centrifuge for ~3-4Hrs. Sample were resuspended in 0.1% TFA.

2.7.2.9. SILAC analysis by MS/MS

Samples were analysed by LC-MS/MS using an ABSciex QStar Elite mass-spectrometer. Peptides were identified using ProteinPilot (Applied Biosystems).

Chapter 3

Identification of potential novel regulators of wild-type and mutant p53

3. Identification of potential novel regulators of wild-type and mutant p53

3.1 Introduction

p53 is a transcription factor with a critical role in preventing tumourigenesis (*8*). When a cell becomes stressed due to factors including DNA damage and oncogene activation, p53 is activated leading to cell cycle arrest and repair, or apoptosis if the stress is severe enough (*8*). This acts to remove damaged or oncogenic cells from replication. In its normal state it functions as a tumour suppressor but can also be considered a proto-oncogene as mutation frequently leads to oncogenic gain of function as well as loss of normal function (*22*). Due to these facts p53 is considered an attractive therapeutic target. As discussed in Chapter 1 a number of strategies have been developed to target p53 in cancer. Which strategy would be implemented will be at least partly dependent upon the mutational status of the tumour. Ways to target p53 include activation of wild-type p53 (wtp53), reactivation of mutant p53 (mtp53) and suppression of mtp53.

In cancers that retain wtp53 it will, in many cases, be beneficial to activate the pathway to bring about senescence or death. Many classic chemotherapeutics bring about p53 activation as a result of DNA damage however this can lead to significant toxicity to normal cells. It has been suggested however that if p53 is activated without initiating DNA damage then toxicity will be much higher in cancer cells than in normal cells. Empirical evidence suggests this may be true, for example a number of MDM2 inhibitors which activate p53 have been shown that they have greater toxicity to cancer cells; xenograph experiments have shown MDM2 inhibitors can lead to tumour shrinkage without substantial toxic side effects (*106, 147*).

In cancers with mtp53 the best option therapeutically may be to reactivate p53. If a therapy can be found that induces mtp53 to achieve its normal confirmation this could both remove the oncogenic gain of functions associated with the mutant conformation and also restore some wild-type functionality. Proof-of-concept experiments have shown that many p53 mutations lead to temperature sensitive changes; when the temperature is lowered p53 can gain normal

confirmation and become active (*148*). Such changes have also been achieved using second-site suppressor mutations or by the introduction of peptides which help to lock p53 in a normal conformation (*121-123*). There has been substantial research to identify chemical therapeutics which could be used to gain such reactivation and a number of promising compounds have been identified (*124-127*).

Whilst reactivating p53 is an attractive therapeutic option it may not be feasible in all cases. Some mutation of p53 may lead to extensive derangement and produce a protein that could not be refolded. Empirical evidence supports the idea that the amount of p53 in the normal or unfolded conformation varies between different mutations (*51*). An alternative to the reactivation approach would be to simply reduce the amount or activity of mtp53 in the cell. Experiments have shown that in tumour cells with mtp53 they are often dependent on it. It has been shown that siRNA mediated knockdown of mtp53 leads to reduced cell growth and apoptosis (*149*) (*52*). This provides proof of concept that compounds that reduce mtp53 levels may be therapeutically useful.

This study will focus on identifying factors which cause the activation of wtp53 and factors which promote the reduction of mtp53 protein levels. This will be addressed by 3 separate screens;

- Screen 1: Screening of small molecules that can activate p53 using cells with a p53-β-gal reporter construct.
- Screen 2: Screening of polymer and hydropolymer matrices that can activate p53 using cells with a p53-β-gal reporter construct.
- Screen 3: Screening of small molecules that can reduce mtp53, assessed by in cell western.

The screens will be presented as three separate studies but overall results will also be concluded at the end of this chapter.

3.2 Screen 1: Screening for small molecules that activate wtp53

p53 is thought to be mutated in around half of all cancers (*8*). This clearly demonstrates the importance of p53 in preventing cancer however it means that at

least 50% of tumours retain wtp53, be it in a homozygous or heterozygous state. In certain cancer types p53 is not typically seen to be mutated e.g. melanoma (*67, 150, 151*). In cancers where wtp53 is present its function is usually supressed by an alternative mechanism in order to circumvent death signals and promote cell survival. For example ARF is frequently lost in melanoma, ARF normally binds to MDM2 preventing its ubiquitination of p53 thus its loss promotes MDM2 and inhibits p53 (*152*). A number of different pathways have been identified in cancer cells which are able to interfere with the normal activation of wtp53. As activation of wtp53 is considered to be therapeutically beneficial any pathways that prevent or promote the activation of p53 could represent potential targets.

The aim of the first screen in this chapter is to identify small molecule inhibitors able to promote the activation of wtp53. This screen utilised cells containing a p53 responsive reporter construct in order to screen for drugs capable of activating wtp53 (Work flow is shown in Fig 3.1). Similar cells have previously been used to successfully screen chemical libraries for drugs which can modify the p53 pathway. Kravchenko et al. (2007) used A431 cells (mtp53) with a β-gal reporter gene under the control of multiple p53 binding elements to screen for chemicals able to reactivate the mtp53 pathway (*146*). They successfully identified RETRA, a drug which leads to an increase in p53 pathway genes via its effects on the p53 family protein; p73. Lain et al. (2008) used T22 cells with a β-gal reporter gene under the control of a ribosomal gene cluster promoter (p53 responsive) to identify compounds able to activate wtp53 (*117*). This led to the identification of Tenovin, a SirT1 and SirT2 inhibitor, as an activator of p53.

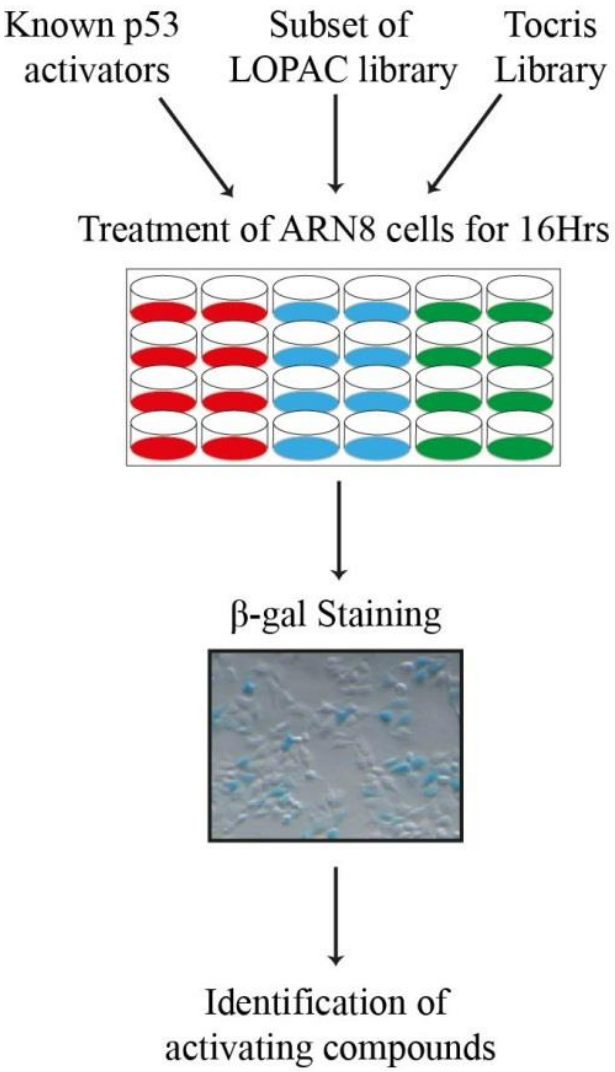

Fig 3.1 Work-flow for Screen 1. ARN8 cells were seeded in 24 well plates and treated with: 1. Known activators of p53, 2. A subset of the LOPAC library and 3. All compounds in the Tocris kinase inhibitor library. Cells were treated with each compound and were tested at 8 different concentrations ranging from 0.4 µM- 50µM and incubated for 16Hrs. Cells were then β-gal stained in order to identify compounds activating p53.

3.2.1 Results

3.2.1.1 Validating the assay

This screen utilised a human melanoma cell line (A375) containing a stable p53 dependent β-gal reporter construct known as ARN8 cells. This cell line was selected as they contain wtp53 which has been shown to be activatable by a number of mechanisms including MDM2 inhibition (*153*). As p53 is rarely mutated in melanoma (*67, 150, 151*) this cell line would be representative of a type where p53 activation could be advantageous.

The work-flow for this screen is shown in Fig 3.1. The cells were treated with 8 different concentrations of each drug ranging from 391nM to 50µM for 16Hrs. They were then β-gal stained in order to detect any p53 activity. These cells contain a *lacZ* gene attached to a p53 responsive ribosomal gene cluster (RGC) promoter. To test for p53 activation the compound x-gal can be added to these cells, if p53 is activate β-galactosidase will be expressed from the *lacZ* gene which will cleave X-gal leading to the production of a blue precipitate. This will result in the cells appearing to turn blue which is observable under a standard microscope. Fig 3.2 shows an example of positive β-gal staining of a p53 activating compound hit. Cell death as a result of inhibitor treatment was not specifically tested for, however when inhibitors led to loss of, or damage to, the cell monolayer this was noted. This was due to the fact that if most cells have detached from the surface then clearly β-gal staining is not possible and thus a negative result cannot be recorded. Also the contraction and detachment of cells could be indicative of cell death and so it is conceivable that apoptosis and p53 activation preceded this state.

In order to assess the validity of this assay, cells were treated with 3 known activators of p53 as positive controls as well as DMSO as a negative control. The three activators used were LMB, Nutlin and Roscovitine. These three drugs activate p53 via different mechanisms. LMB is a nuclear export inhibitor which prevents p53 from exiting the nucleus. As p53 is a transcription factor it needs to be localised to the nucleus to function, LMB therefore allows p53 to stay active by prohibiting its nuclear exit (*114*). Nutlin is an inhibitor of the p53 negative regulator; MDM2.

Nutlin prevents the binding of MDM2 to p53, thus preventing MDM2 mediated ubiquitination and subsequent degradation of p53 (*108*). Roscovitine is a Cdk inhibitor which causes the activation of p53 via multiple systems including repression of MDM2 and disruption of the nucleolus (*154, 155*).

As detailed in Table 1 all three compounds produced strong p53 activation at a range of concentrations. It was observed that none of these drugs appeared to cause substantial cell loss from the plate compared to DMSO. These results suggest that β-gal staining of ARN8 cells is capable of correctly identifying compounds which activate p53 via a number of different upstream mechanisms. It was therefore considered that this assay could be used to screen compounds to identify novel activators of p53

3.2.1.2 LOPAC library compounds

An initial pilot screen completed in the lab (*data not published*) identified a number of compounds from the LOPAC library that appeared to activate p53. The compounds identified in that screen, which are detailed in Table 3.3, were assessed in this screen. As shown in Table 3.1, only 3 of the 16 compounds tested activated p53 in this screen. These were Apigenin, Aminopterin and Z-L-phe chloromethylketone. As an example of the typical staining observed, Figure 3.2 shows the presence of blue cells (activated p53) that can be seen with Apigenin. Both Apigenin and Aminopterin did not appear to lead to cell death after 16hrs as the number or morphology of cells did not obviously appear to be affected. Z-L-Phe chloromethylketone however did appear to cause loss of cells from the plate surface (Table 3.1).

3.2.1.3 Kinase inhibitor library

Kinases are critical in the regulation of many cellular mechanisms. p53 is heavily regulated by various kinases and has been shown to have >20 phosphoacceptor sites (*156*). Furthermore, kinases are considered good drug

targets and are considered an important group in the “drugable genome” (*157*). Therefore, this screen was also used to test a kinase inhibitor library (Tocris Kinase inhibitor toolbox) for inhibitors that can activate wtp53. This library contains 80 compounds targeting 35 kinases which are detailed in the supplementary data (Table S1). The results of this screen are shown in Tables 3.1 and 3.2. Screening of the kinase library identified 9 inhibitors which were able to activate p53. These represent 7 specific targets as two inhibitors target Cdk and 1 inhibitor (Ro-31-8220) is a broad spectrum inhibitor. Two of the inhibitors; Aminopurvalonol A and Ro-31-8220, a Cdk and broad spectrum inhibitor respectively, showed disruption of the cell monolayer at the higher concentrations with activation of p53 at lower concentrations. This disruption is likely due to cell death although this was not specifically assessed. It could be speculated that such cell death may be due to p53 mediated apoptosis, however this remains to be confirmed. A number of the inhibitors, which did not stain positive for p53 activation, also appeared to lead to substantial monolayer disruption; again however, it remains undetermined whether p53 played a role in this.

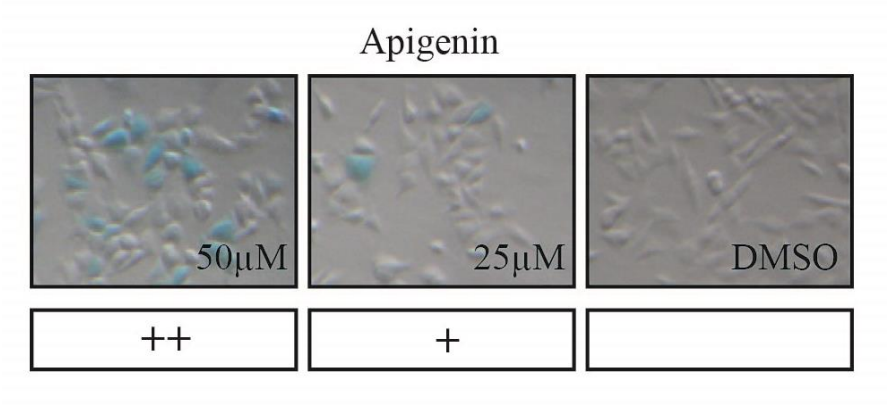

Fig 3.2 Apigenin activates p53 in ARN8 cells. ARN8 cells were treated with the indicated concentration of Apigenin and then β-gal stained in order to assess p53 activity. The strength of activation was assigned qualitatively and are indicated in the boxes below.

Table 3.1. Compounds identified as activators of p53 in Screen 1. ARN8 cells containing a p53 β-gal reporter construct were treated with various drugs (detailed in 3.2.1.1, Table 3.3 and Table S3) at 0.4μM-50μM for 16Hrs, p53 activation was then assessed by β-gal staining. Relative strength of activation was determined qualitatively based on the number of blue cells (++ = high, + = low). ☠ indicates substantial disruption/loss of the cell monolayer (assessed by eye). If no p53 activation or loss of cells were observed at a given concentration the grid cell is left blank. Drugs are only contained in this table if any p53 activation was observed. DMSO was tested as a control but did not lead to p53 activation and so is not included within this table.

Drug	**Target**	**50μM**	**25μM**	**12.5μM**	**6.3μM**	**3.1μM**	**1.6μM**	**0.8μM**	**0.4μM**
Roscovitine	Cdk	++	++	++					
Nutlin	MDM2	++	++	++	++	++	++		
LMB	CRM1	++	++	++	++	++	++	++	
Apigenin	Broad spectrum	++	+						
Aminopterin	DHFR			++	++	++	++		
Z-L-Phe chloro-methylketone	Chymo-trypin	☠	☠	☠	++	++	++	++	++
Fasudil Hydrochloride	ROCK	++							
GF109203X	PCK	+	+						
Genistein	EGFR	+	+						
Olomucine	Cdk	+							
PP1	Src		+						
Purvalanol A	Cdk	☠	☠	+					
SB415286	GSK3	+	+						
Ro 31-8220 mesylate	Broad spectrum	☠	☠	☠			++	++	+
Dorsomorphin dihydrochloride	AMPK			++	+				

Table 3.2. Compounds leading to disruption of ARN8 cell monolayer in Screen 1. ARN8 cells containing a p53 β-gal reporter construct were treated with various drugs (detailed in 3.2.1.1, Table 3.3 and Table S3) at 0.4μM-50μM for 16Hrs. p53 activation was then assessed by β-gal staining (Results detailed in Table 3.1). Additionally cells were visually checked for substantial disruption/loss of the cell monolayer. Detailed within this table are drugs which caused only cell monolayer disruption, this is indicated by ☠. If no loss of cells were observed at a given concentration the grid cell is left blank. DMSO was tested as a control but did not lead to monolayer disruption and so is not included within this table.

Drug	Target	50μM	25μM	12.5μM	6.3μM	3.1μM	1.6μM	0.8μM	0.4μM
ZM449829	JAK3	☠							
ZM39923 Hydrochloride	JAK3	☠	☠						
Amino-purvalanol A	Cdk	☠							
BIBX 1382 dihydrochloride	EGFR	☠							
CGP 53353	PKC	☠							
IKK16	IKKβ	☠	☠						
10-DEBC hydrochloride	PKB	☠	☠						
CGK 733	ATR/ATM	☠	☠						
PHA 665752	cMET	☠	☠	☠					
PD 407824	Chk1	☠							
PQ 401	IGF-1R	☠	☠						
EO 1428	p38 MAPK	☠							
H89 dihydrochloride	PKA	☠							
BIO	GSK3	☠	☠						
BI 78D3	JNK	☠	☠	☠					

Table 3.3. LOPAC library compounds tested for ability to active p53. Descriptions supplied with information from manufacture (Sigma).

Drug	Target	Action	Class	Description
Aminopterin	Dihydrofolate reductase	Inhibitor	Antibiotic	Dihydrofolate reductase inhibitor
Apigenin		Inhibitor	Cell Cycle	
Chlorzoxazone	iNOS	Inhibitor	Nitric Oxide	Inducible nitric oxide synthetase inhibitor; skeletal muscle relaxant
Psora 4	Kv1.3	Inhibitor	K+ Channel	Potent Kv1.3 potassium channel inhibitor.
ATPA	Kainate	Agonist	Glutamate	Selective kainate glutamate receptor agonist
SKF-89145 hydrobromide	D1	Agonist	Dopamine	D1 dopamine receptor agonist
Tracazolate		Modulator	GABA	Non-benzodiazepine anxiolytic which has a high differential between sedative and anxiolytic doses
9-cyclopentyladenine monomethane-sulfonate	Adenylate cyclase	Inhibitor	Cyclic Nucleotides	Cell-permeable, non-competitive adenylyl cyclase inhibitor; targets the P-site domain
L-(-)-norepinephrine (+)-bitartrate salt monohydrate		Agonist	Adrenoceptor	Adrenergic neurotransmitter
AraC		Inhibitor	DNA Metabolism	Selective inhibitor of DNA synthesis
SB200646 hydrochloride	5-HT2C/2B	Antagonist	Serotonin	5-HT 2C/2B serotonin receptor antagonist.
Z-L-Phe Chloromethyl Ketone	Chymotrypsin A-gamma	Inhibitor	Biochemistry	Bovine chymotrypsin A-gamma inhibitor
Org 24598 lithium salt	GlyT1	Inhibitor	Neuro-transmission	Selective, potent inhibitor of glial GlyT (GlyT1, glycine transporter type 1).
2-azetidine-2-carboxylic acid	Collagen	Inhibitor	Biochemistry	Inhibits collagen synthesis
acetamide	Carbonic anhydrase	Inhibitor	Biochemistry	Carbonic anhydrase inhibitor; diuretic
sandoz 58-035	ACAT	Inhibitor	Lipid	Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor

3.2.1.4 Preliminary validation of four activators of p53

In the above experiments 15 compounds were shown to activate p53 in the β-gal assay. A number of these represent drugs which have previously been shown to be able to activate p53 which supports the validity of the assay. Apigenin, Aminopterin, Olomucine, Roscovitine, Nutlin and LMB have all been extensively shown (or the class of drug shown) to lead to p53 activation (*108, 114, 154, 155, 158-161*). The remaining compounds represent either novel activators of p53 or compounds that have previously been shown to activate p53 but where the evidence is more limited. In either case, this screen can potentially help increase our understanding of the importance of the drug targets contribution to the p53 pathway. Not all 15 compounds could be further investigated within the confines of this study but 4 compounds were selected for preliminary validation. The drugs selected were Dorsomorphin dihydrochloride, SB-415286, GF109203X and Genistein which are described as AMPK, GSK3, PKC and EGFR inhibitors respectively.

When p53 is activated it escapes degradation and is often seen to be stabilised within the cell (*162*). As a result increased p53 protein within the cell could be indicative of p53 activation. If the p53 is activated this should be coupled with a rise in the levels of p53 responsive genes including MDM2 and p21 (*163*). In order to validate the results, cells treated with Dorsomorphin dihydrochloride, SB-415286, GF109203X and Genistein were analysed by western blot for the effect of the drug on p53, MDM2 and p21 protein levels. As can be seen in Fig 3.3 all 4 compounds show some stabilisation of p53, MDM2 and p21. Dorsomorphin dihydrochloride lead to an increase in all three p53 pathway genes in a concentration dependant manner. SB-415286, GF109203X and Genistein also showed a general increase with increasing drug concentration however small fluctuations were seen in MDM2, p21 and p53 meaning that a strict concentration dependent effect is not observed. Activation of p53 is known to increase the levels of MDM2 however MDM2 in turn acts to negatively regulate p53 forming a negative feedback-loop. This feedback loop leads to cycling fluctuations in the levels of both p53 and MDM2 (*164*). Similarly p21 levels are also known to fluctuate due to

regulatory feedback (*165*). The apparent lack of adherence to a strict concentration dependent effect likely reflects where in this fluctuating cycle proteins were at the time of harvesting.

Overall the increased levels of p53, MDM2 and p21 suggest that p53 is being activated by the compounds, supporting the results of the β-gal screen and suggesting that SB-415286, Dorsomorphin dihydrochloride, GF109203X and Genistein can lead to the activation of p53 in A375 cells.

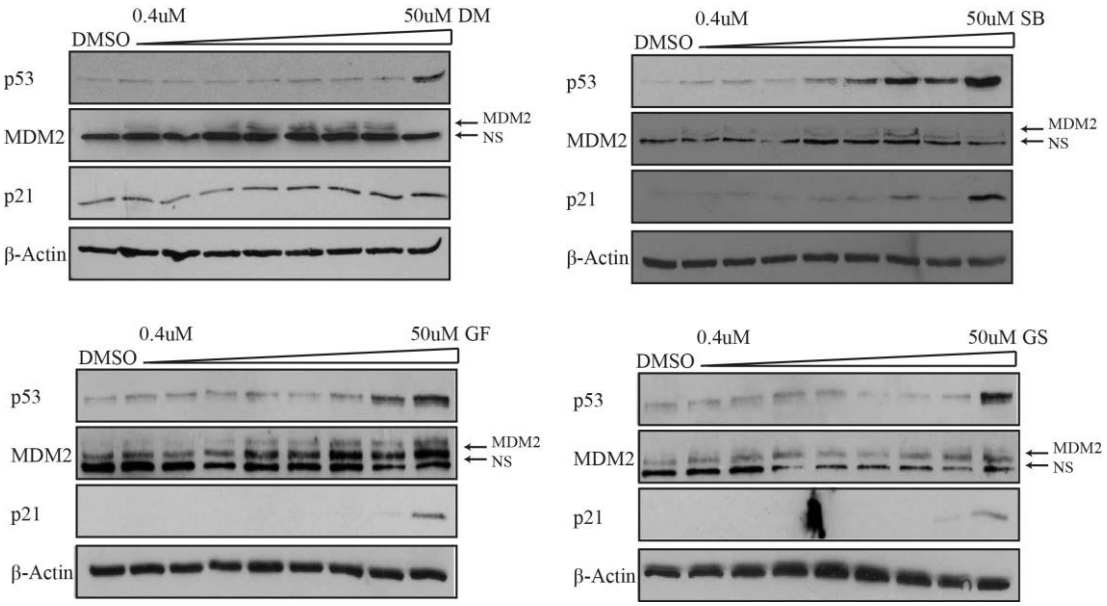

Fig 3.3. Activation of p53 by Dorsomorphin, SB-415286, GF109203X and Genistein. A375 cells were treated with (A) Dorsomorphin, (B) SB-415286, (C) GF109203X and (D) Genistein for 16Hrs. Cell lysates (20µg/well) were then assessed by western blotting to determine relative levels ofp53, MDM2 and p21 using anti-p53 (DO1), anti-MDM2 (2A10) and anti-p21 antibodies. Equal loading was also assessed using anti-β-actin antibody.

3.2.2 Discussion

This screen successfully identified a number of inhibitors which are able to activate p53. The rationale for the subset of LOPAC library inhibitors tested was based on a previous screen conducted in the lab (*data not published*). This pilot screen used the same cell type with a p53 dependent β-gal reporter construct (ARN8 cells) to screen the LOPAC library. This current screen was used to attempt to replicate the results of the hit compounds. The replication of results of the previous screen in the lab did not show strong consistency, with only 19% (3/16) of results being replicated. This level of reproducibility is surprisingly low but highlights the need for results to be further validated. p53 is a stress related protein which can be strongly affected by numerous factors (*8*). If cells are being grown in sub-optimal conditions then p53 may be much more likely to activate by additional stressors or drugs.

Two of these hits confirmed from the previous screen, Apigenin and Aminopterin, have previously been shown to activate p53 further validating the effectiveness of this screen. A derivative of aminopterin, methotrexate, is a commonly used chemotherapeutic used in the treatment of a range of cancers. These drugs inhibit dihydrofolate reductase an enzyme involved in the production of purines, thymidylic acid, and certain amino acids and thus important in DNA synthesis and repair. Inhibition of this enzyme causes phosphorylation of p53 at ser-15 and acetylation at Lys373/382 (*159*). Acetylation and phosphorylation of p53 can both increase the activity of p53 by preventing the interaction with MDM2 or enhancing its ability to bind DNA (*18*).

Apigenin is a plant derivative which is thought to have potentially useful chemotherapeutic and chemopreventative properties. Apigenin leads to the activation and increased stability of p53 via phosphorylation and a decrease in MDM2 and can lead to p53 dependent apoptosis (*160*). Whilst these drugs do not represent novel activators of p53 they provide confidence that the assay is able to detect such activators.

The final set of drugs tested in this assay was the Tocris kinase inhibitor toolbox. Kinases are enzymes which transfer phosphate groups from donor molecules such as ATP to their substrates. There are thought to be >500 kinases present in the human cell and each has a specific and limited number of substrates (*166*). Phosphorylation often directly affects the activity and localisation of its substrates and kinases orchestrate the activity of virtually all cellular processes (*167*). Critically, deregulation of kinase activity is often a major mechanism by which cancer cells survive and grow (*168*). Inhibition of a specific kinase could therefore prevent or enhance a process which is critical or detrimental to only cancer cells. There are a growing number of kinase inhibitors which have been approved for clinical use(*168*). The use and further development of kinase inhibitors is particularly attractive in view of the fact that conventional cancer treatment often utilises chemicals which lead to DNA damage in all cells and relies on the fact that cancer cells die faster than normal cells (*169, 170*). The problem comes when selecting which kinases of the >500 present in cells would be an appropriate target. Screening therefore provides a useful mechanism by which kinases important to the pathway of interest, in this case p53, can be identified.

Screening of the Tocris kinase inhibitor toolbox revealed 9 compounds that lead to positive β-gal staining. Once again some of the inhibitors identified have previously been shown to activate p53. One of the hits, olomucine, has been established to activate the p53 pathway in certain cells (*171*). Olomucine is a Cdk inhibitor from which roscovitine is derived. A third Cdk inhibitor aminopuvalanol A was also identified by the screen. Cdks are a family of kinases which are important in cell cycle progression as well as the control of transcription. Roscovitine and olomucine have been shown to most strongly inhibit cdks 1, 2 and 5 which are involved in cell cycle progression although they have also been shown to repress transcription which may be mediated by inhibition of cdk 7 / 9 (*172*). Whereas aminopurvalanol A is reported to most strongly inhibit cdks 1 and 2 (*172*). Aminopurvalanol A however has not been as widely characterised as Roscovitine/olomucine. Cdk inhibition can lead to activation of p53 by a number of

mechanisms including inhibition of MDM2 and disruption of the nucleolus (*154, 155*). p53, however, appears not to be required for cell death mediated by roscovitine/olomucine as such inhibitors appear to have multiple mechanisms of action (*173*). Nonetheless Cdk inhibitors are thought to be an attractive therapeutic target and a number are currently in clinical trials (*174*). p53 is not thought to be strictly necessarily for the anticancer effects of cdk inhibitors however the anticancer effects of some are enhanced in the presence of wtp53 (*173*).

A number of the compounds identified represent potentially novel modifiers of the p53 pathway however further work would be required in order to confirm these results. Nonetheless, these results represent promising leads for future analysis. Within this study four drugs were selected for preliminary validation; GF109203X, Dorsomorphin dihydrochloride, Genistein and SB-415286. All four of these drugs showed the ability to activate p53 by the β-gal reporter assay and by western blotting which enhances confidence in the initial results of the screen. However the observed activation by both the β-gal reporter assay and by western blotting with all four drugs was at relatively high concentrations. This increases the need for further research to show whether or not p53 activation results from on-target effects. However there is evidence in the literature to support the idea that inhibition of some of these targets could activate p53 and will be discussed now.

GF109203X is a PKC inhibitor. PKC is a family of kinases which are important in the transduction of many signalling pathways. There are 10 isoforms of PKC with numerous targets and roles in various pathways including receptor desensitisation, transcription, immune response and cell cycle regulation. The outcome of PKC inhibition is variable and different isoforms have been shown to have differential effects on p53 with some being inhibitory and others enhancing its activity (*175*). The effect of inhibition will also be effected by the level of expression of each isoform within the cell as some PKC isoforms have been shown to be overexpressed in cancer (*176*). GF109203X is an PKC inhibitor with highest activity towards PKCα (*177*). PKCα has been shown to be overexpressed in some cancers and consistent with the findings here, at least in certain circumstances, PKCα inhibition has been

shown to lead to reduced proliferation and p53 activation (*176*). The molecular mechanism of PKCα pro-proliferative role is not clear but may involve the phosphorylation and activation of antiapoptotic proteins Bcl-2(*178*), and Akt (*179*).

Dorsomorphin is an AMPK inhibitor; AMPK is primarily thought to be involved in energy homeostasis. Conversely to the findings presented here AMPK activators are being explored as a cancer therapy and it has been shown that AMPK activation in some cases can induce p53 dependent apoptosis (*180*). However some studies have, in agreement with the findings here, found inhibition to increase apoptosis. For example it has been shown that AMPK protects cisplatin treated cells from apoptosis and AMPK inhibition can synergise with cisplatin to induce p53 dependant apoptosis (*181*).

Genistein was also shown to activate p53 by western blot and β-gal staining. Genistein is an isoflavone which is naturally occurring in a number of plant food sources. It is primarily known as an inhibitor of EGFR however it also affects a number of other pathways including activation of PPARs, inhibition of topoisomerase and activation of beta oestrogen receptors (*182, 183*) as such its ability to activate p53 cannot specifically be assigned to inhibition of EGFR. As Genistein affects a number of systems the direct and indirect effects from subsequent cross talk are complex. Consistent with the findings here Genistein has been shown in the literature to activate p53. This may occur as a result of changes to a number of pathways including repression of MDM2 and positive regulation of the ATM and PTEN pathways which can act to promote p53 (*184-186*).

Finally a GSK-3 inhibitor; SB-415286 was shown to activate p53. GSK-3 is a serine/threonine kinase which has a diverse range of roles including in glycogen metabolism, cell cycle regulation and apoptosis. Depending on experimental conditions GSK-3 appears to have contradictory roles in apoptosis and can act as both a pro or anti-apoptotic factor. GSK-3 is a target of interest in many neurodegenerative diseases and its inhibition has been shown to reduce neuronal cell death (*187*). In neuroblastoma cells it was shown that the apoptotic effect of staurosporine treatment was at least partially dependant on GSK-3

(*188*). Conversely in many other cancers GSK-3 has been shown to have an anti-apoptotic effect and may be a potential therapeutic target. Ghosh et al. (2005) showed that GSK-3 inhibition maybe a potential target in colorectal cancer. They showed that chemical inhibition, siRNA and dominant negative GSK3 lead to p53 dependent apoptosis (*189*). This anti-apoptotic effect of GSK-3 may be mediated by a number of mechanisms. GSK-3 phosphorylates MDM2 thereby promoting the degradation of p53 (*190*) and as such GSK-3 inhibition could allow p53 to escape degradation. Furthermore GSK-3 has also been shown to positively regulate NF-kB which, in some cases, protects cells from apoptosis (*191*).

The relatively large number of compounds identified from this small scale screen could reflect the large number of pathways involved in p53 control (*22*). However it has been suggested that the conditions in cell culture make p53 activation more likely and could lead to responses that would not be seen in a whole organism (*192*). Whilst this does emphasise the need for further testing, ideally in a whole organism, this limitation is difficult to get around in a first line screen. Normal tissue is also likely to react differently to cancer tissues and the testing of a large numbers of compounds in tumours growing in animal models is impractical.

In conclusion, this screen has found a number of inhibitors that appear to be able to activate p53. Some of these represent potentially novel activators of p53 although further work will be needed to confirm this activity. However, four of the compounds were confirmed to be activators of p53 by western blotting in this study. Future work on these compounds would need to explore the mechanism of this activity and establish whether perturbation of the kinase target i.e. by RNAi could also lead to p53 activation.

3.3 Screen 2: Tissue engineering approach to p53 activation

Cell signalling occurs both within a cell and between cells over both small and large distances. It is also apparent that cells can be altered as a result of other materials in, or introduced into, their environment (*193, 194*). From an immunological perspective it is vital that any material or prosthesis introduced in

the body is relatively inert. However some changes in cells induced by various materials can be exploited by medical and biological science in order to manipulate certain activities. If cells are grown in contact with biosynthetic materials then the cells can be altered by such contact (*193-195*) and this is exploited in the field of bioengineering.

The ability of biosynthetic materials to support and manipulate cell growth is controlled by both the chemistry and topology of such materials (*195*). Research has shown that by altering these factors a diverse range of cellular responses can be manipulated, these include cell adhesion, growth, survival and differentiation (*193, 194, 196*). Testing of polymers to identify those which achieve the desired outcome can be a slow and iterative process and the desires to move towards high-through put screening has led to the development of polymer arrays (*197*). Such arrays typically consist of spots of different polymers arrayed on glass slides allowing hundreds of polymers to be screened in one experiment.

One example of intensive biopolymer research where arrays have been utilised is within bone regeneration. Research is aiming to identify polymers which can be injected into a site where bone has been lost to provide a scaffold for bone growth. The ideal biomaterial will promote bone cell differentiation and growth and provide a surface to direct the formation of the new tissue. Khan et al. (2010) screened a range of polymer blends for those that would support growth and a number of blends were successfully identified. Further analysis of one of these blends showed that it supported growth of bone progenitor cells and initiated early differentiation. *In vivo* models showed that the scaffold enhanced bone regeneration (*193*).

A further example of the use of polymer microarrays is the study by Hansen et al. (2011). They screened polymers on a microarray to identify polymers capable of causing the activation and aggregation of platelets (*194*). They were successful in identifying a number of polymers and suggested that they could be used to coat bandages to quickly staunch blood flow in trauma victims.

The effect of biomaterials on p53 signalling however, has been investigated largely in terms of understanding any oncogenic effects of materials to be used in the body. Clearly a material that was particularly suppressive to p53 may represent a carcinogenic risk whereas a material which strongly promoted p53 may inhibit wound healing. Van kooten et al. (2000) showed that in umbilical cord derived cells, grown in the presence of various metals, p53 levels increased after around 48hrs and was negatively associated with the proliferation marker Ki67 (*198*). This illustrates the point that proteins important in the cell cycle can be affected by growth surface. Bombonato-Prado et al. (2007) did a more extensive analysis of various different materials including glass, stainless steel, polymethylmethacrylate (PMMA) and titanium (*199*). Using microarray analysis they showed that both PMMA and stainless steel led to a reduction in p53 mRNA. Whilst any material that strongly suppresses or promotes p53 may be harmful to the body it could potentially provide a useful tool to study p53 pathway control.

The aim of this study is to screen arrays to identify polymers which can affect the activation of p53. Such polymers would provide an extremely useful research tool. In order to study the p53 pathway in an active state research frequently relies on the addition of drugs. If p53 activating polymers were identified, cells could be grown on plates coated with such polymers to activate the pathway. As well as providing an alternative to small molecules this could provide a number of other advantages. Plates could be half coated in the material or coated in a gradient concentration and cells grown over the surface. The effect of activation could then be studied by techniques such as in cell western or microscopy within one well. This would remove any uncontrolled variable effects of using multiple wells such as differences in cell density and microenvironment.

3.3.1 Results

3.3.1.1 Polymer and hydropolymers arrays

The use of polymer arrays was developed in part by Mark Bradley's group (*200*). Two types of arrays previously used by the group were selected for testing to

identify polymers able to activate p53. The 2 categories of arrays used are from here on referred to as polymers and hydropolymers arrays. The hydropolymers and polymer arrays were produced by Rong Zhang and Mei Wu (Mark Bradley Lab, Edinburgh University) respectively. Details of the production of the polymer arrays can be found in Tourniaire et al. (2006) (*200*) and details of the specific polymers used can be found in Wu et al. (2012) (*201*). Arrays were composed, as described, of 652 polymers in triplicate, including 468 polyacrylates and 164 polyurethanes. Polymer arrays were made by the contact printing of pre-synthesised linear polymers. Polymer arrays were spotted on slides coated with agarose which aids contact printing but does not support cell adhesion.

Details of the production and polymer types in the hydropolymer arrays can be found in Zhang et al. (2009) (*196*). Hydropolymer arrays were composed of 609 polymers in quadruplicate composed of different combinations of the 19 monomers described in Zhang et al. (2009) (*196*). The arrays used within this experiment differed slightly from those described in that fewer combination ratios of the described monomers were used. As detailed hydropolymer arrays were produced by printing of monomers which were polymerised in situ. To construct the hydropolymer arrays slides were first printed with a 20% sucrose aqueous solution to form a sugar array which was then treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-dimethylchlorosilane (fluorosilane). This surface allows cellular binding and so cells could adhere to the entire slide. Monomers were then printed onto the plates and polymerised in situ.

The work-flow for this screen is shown in Fig. 3.4 A. To analyse the effect of each polymer and hydropolymer on p53, ARN8 cells (containing a p53 responsive β-gal reporter gene) were grown on the arrays and reporter expression was analysed. In order to observe β-gal staining each polymer spot was photographed and then images were manually checked. The polymers were screened over a time course of 6, 12, 24, 48 and 72Hrs. The hydropolymers were screened at only one time point due to the limited number of hydropolymer slides available.

The hydropolymers were also tested for their ability to repress p53 activated by roscovitine treatment. The work-flow for this screen is shown in Fig 3.4 B. Hydropolymer spots were again individually photographed and were this time analysed to identify spots which had reduced β-gal staining. Roscovitine was selected to activate p53 as it is known as a strong activator of p53. It was validated to cause observable p53 activation in ARN8 cells in the previous screen (Table 3.1).

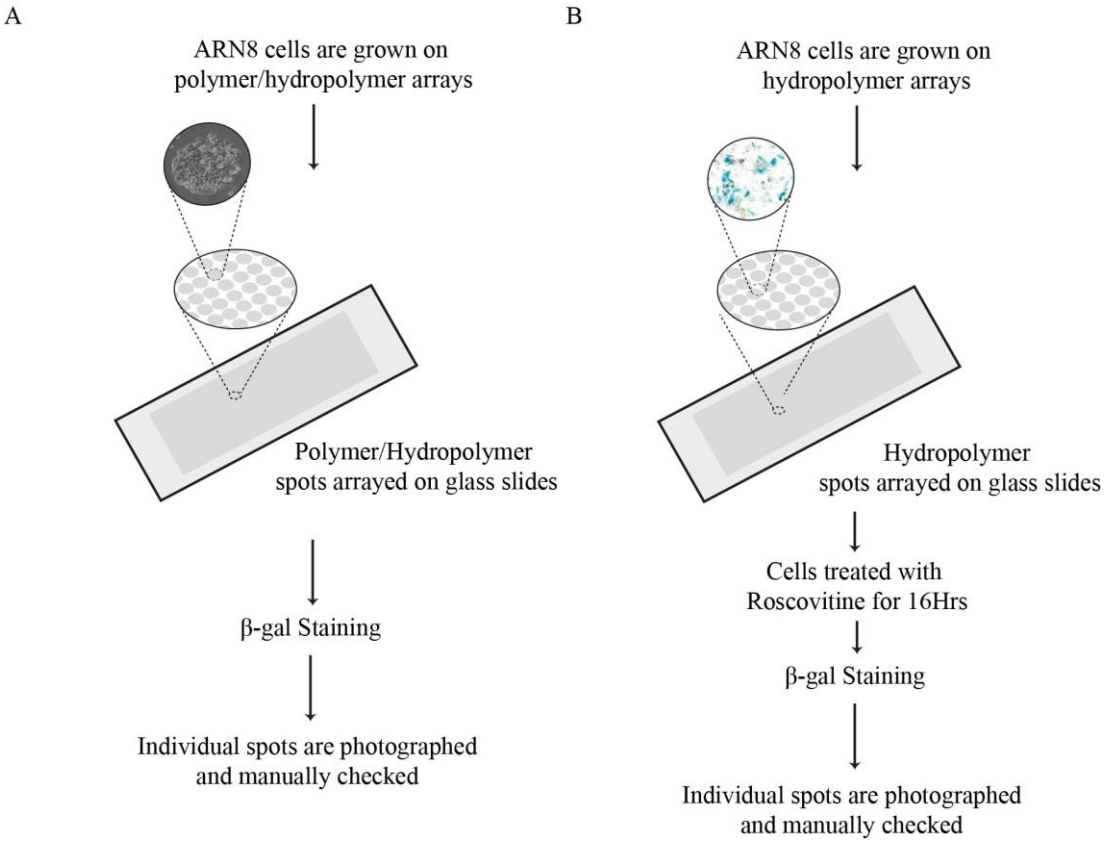

Fig 3.4 Work-flow for Screen 2. (A) ARN8 cells were grown on polymer/hydropolymer slides. The cells were then β-gal stained to identify any polymers/hydropolymers which activate p53. **(B)** ARN8 cells were grown on hydropolymer slides. Cells were treated with Roscovitine for 16Hrs. The cells were then β-gal stained to identify any polymers/hydropolymers which activate p53.

3.3.1.2 p53 activation by polymers

When ARN8 cells were grown over the surface of the polymer slides, as predicted cells were not seen to bind to blank areas of the slide and whilst it was not specifically assessed it was noted that the different polymers appear to affect the adhesion and growth of cells over the surface causing different growth patterns to be observed (Fig 3.5A). The thickness of the polymers over the spot surface may not have been continuous, which could influence cell growth over the surface. However, such changes cannot be due to the printing method alone as if this were the case the same pattern of cells should be observed over every spot. However, unfortunately no p53 activation was seen at any time point as assessed by β-gal staining (Fig 3.5B). Potentially p53 could have been activated for a short time period in some of the spots but the cells died and quickly detached before the next time point. However, as the purpose of this study is to identify a growth surface that will activate p53 in order to use it as a research tool, a short activation window followed by rapid death would not allow much time for any type of analysis or experimentation. This outcome would be desirable when seeking to identify chemotherapeutics but may not provide an ideal research tool. For this reason extra time points over the period were not further investigated.

3.3.1.3 p53 activation by hydropolymers

ARN8 cells were grown over the hydropolymer arrays for 24Hrs and then assessed for β-gal staining. Similarly to the polymer arrays it was noticed that some hydropolymers support better growth than others. However, as with the polymer spots, the hydropolymers also showed no ability to activate p53 (Fig 3.5C).

3.3.1.4 p53 repression by hydropolymers

Hydropolymer slides were then analysed for their ability to repress p53 activation by roscovitine. As can be seen in Fig 3.6 Roscovitine provided an even spread of cells with activated p53. Whilst it does not cause p53 in all cells to become

active, the cells are dense enough for several blue cells to be observed over the surface of each hydropolymer spot. Using this approach meant that hydropolymers that completely or highly repressed p53 activation could be identified, although it would be difficult to observe hydropolymers with weak ability to repress p53. However no hydropolymers were able to repress p53 activation by roscovitine (Fig 3.7).

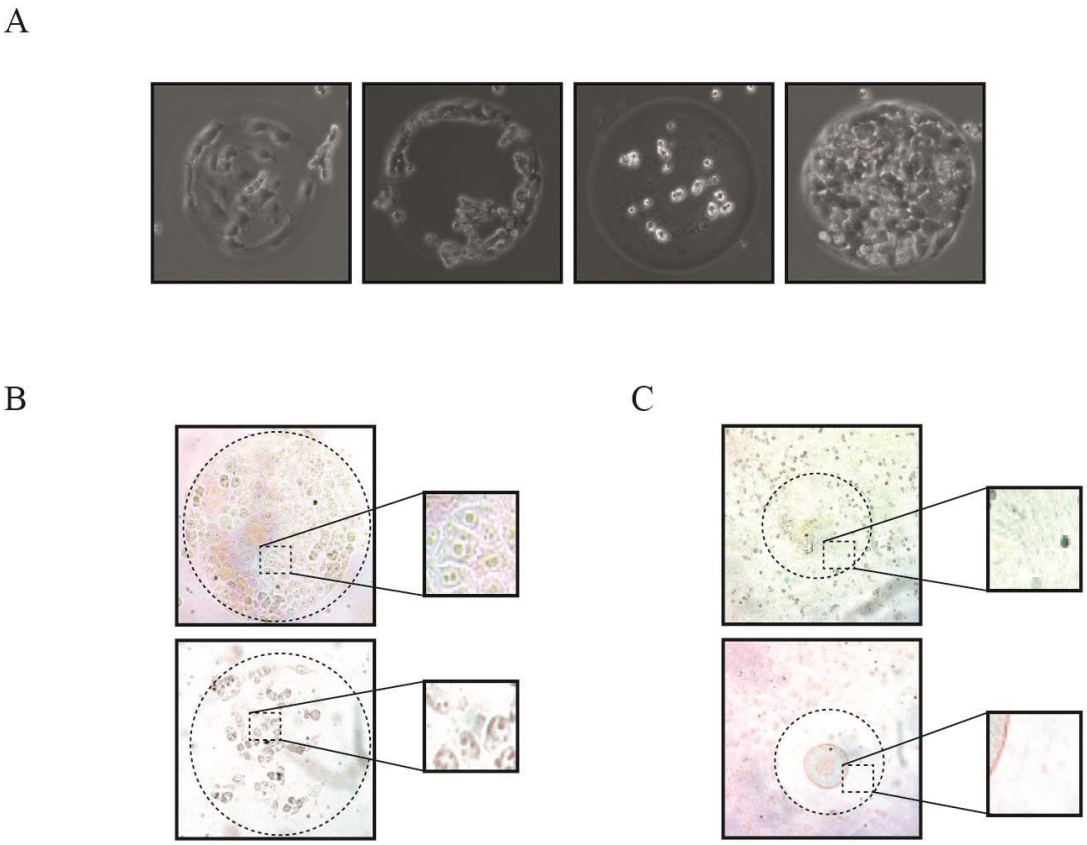

Fig 3.5 No polymers/hydropolymers spots were identified which activated p53. (A) Examples of the different growth patterns observed when ARN8 cells were grown over polymer microarrays. As can be seen some polymers supported cell growth better than others, some restricted this growth to only the outside or central regions **(B)** Examples of β-gal stained ARN8 cells grown on polymer arrays (negative results are shown as no hydropolymers activated p53). Polymers are demarked by circular dashed lines and expanded sections shows the cell attached over this area. **(C)** Examples of β-gal stained ARN8 cells grown on hydropolymer arrays (negative results are shown as no hydropolymers activated p53). Hydropolymers are demarked by circular dashed lines and expanded sections shows the cell attached over this area. The lower panel shows no cells attached over the spot.

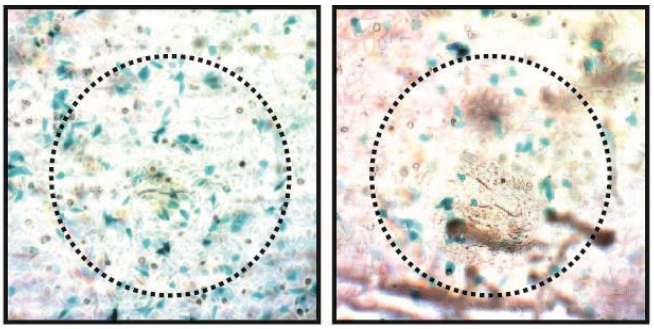

Fig 3.6. No hydropolymers were identified which prevented p53 activation by Roscovitine. ARN8 cells were grown on hydropolymers spots for 24Hrs and treated with Roscovitine for 16Hrs. After treatment they were fixed and β-gal stained to assess p53 activity. Typical examples of hydropolymer spots after staining are shown. Dashed lines demark the hydropolymer spots. The level of activation on the hydro polymer spot was compared to the level of activation in the surrounding area where no hydropolymers were present.

3.3.2 Discussion

This screen aimed to identify polymers and hydropolymers able to regulate the p53 pathway. Polymers were analysed for their ability to cause p53 activation, and this was assessed at a number of time points. Hydropolymers were analysed for their ability to either activate or repress p53 activation 24 hrs after seeding.

Both arrays have previously, or since been, successfully used in other studies. The hydropolymer array was used to identify hydropolymers which allow cell binding and proliferation and subsequently thermal release (*196*). The polymer array was used to identify polymers which inhibited or aided *Cryptosporidium parvum* oocyte binding and assess the effect of hydrophobicity, roughness and polymer composition on the outcome (*201*). Unfortunately however, no polymers in either of these arrays that activated p53 were identified. Growth over the different polymers was observed to vary, however β-gal staining did not indicate that any polymers or hydropolymers lead to the activation of p53.

Polymers have been shown to be capable of greatly altering protein expression and activity bringing about differentiation (*193, 198*). This suggests that polymers could be found that could alter activity of a specific protein. p53 can be activated by affecting any of a large number of mechanisms and furthermore other studies have demonstrated that biomaterials are capable of affecting p53 (*198*).

One explanation may be that polymers that activate p53 may be less likely to support initial growth and so any potential activation is never realised. Due to the fact that p53 is activated by cellular stress it is conceivable that such “stressful” conditions would be less likely to support initial growth. Activation of p53 will typically cause cell cycle arrest or apoptosis (*8*). If signals from the growth surface cause apoptosis clearly few cells would be observed on that surface and so these materials would be difficult to identify. However, biomaterials that increase p53 have been previously identified; Van Kooten et al. (2000) showed that various metals could increase p53 protein level in umbilical cord derived cells (*198*). Although, it should be noted that this study did not specifically investigate activity of

p53. Nonetheless, it does suggest that materials do exist that could allow cell attachment and affect p53.

Hydropolymers were also screened to identify any polymers that were able to repress the activation of p53 by roscovitine. Again, no such hydropolymers were successfully identified. One issue with this study is that roscovitine does not lead to an observable response in all cells, only a proportion. This is an issue as if only a proportion of the cells are activated in the control it makes it much harder to discern subtle differences with the hydropolymers. This means it can be said for certain that no hydropolymers led to the complete repression of activation but reduced activation may not have been identified. In future work it may be beneficial to repeat this with a stronger activator of p53. Alternatively a more quantitative technique could be used to assess any changes. As discussed in the following screen, in cell western is a useful technique to look at protein expression within fixed cells. Future work could use this technique to identify polymers which lead to a decrease or increase in p53 and down-stream proteins such as MDM2 and p21. This would allow a more quantitative approach to assessing p53 activity.

In conclusion, this study suggests that finding a biomaterial which activates p53 is difficult and such materials are not easily identified. This study utilised polymer arrays containing polyacrylates and polyurethanes as well as hydropolymers composed of combinations of 19 monomers. Clearly this only represents a fraction of total possible polymers. In future work it may be useful to use larger arrays as a starting point. This study also failed to identify hydropolymers that repressed the activation of p53 by roscovitine. This study was technically challenging as roscovitine only produced observable activation in a proportion of cells. Further work would be needed to identify either a stronger activator of p53 or an alternative read-out of p53 activity; this would allow hydropolymers that caused more subtle repression of p53 activity to be identified.

3.4 Screen 3: Small molecule screening for drugs able to reduce levels of mtp53

A number of diseases are associated with the aberrant accumulation of specific proteins within the cell. This is also true of many cancers, as mutation in p53 frequently leads to its accumulation within the cell (*202, 203*). Mutation within the p53 gene frequently confers oncogenicity (*32*). The oncogenic capability of mtp53 is mediated by aberrant interaction with a number of proteins including the p53 family proteins; p63 and p73. Mtp53 has been shown to stably interact with p63 and p73 preventing them from activating p53 pathway genes (*36*). Other pathways where mtp53 has been shown to have altered roles include EGR-1, Nf-κB, NF-Y and TGFβ (*43, 47, 49*). Such aberrant interactions have generally been found to enhance anti-apoptotic signals, cell growth and invasion (*22*). Studies have shown that the removal of mtp53 from the cell can inhibit growth or even induce cell death (*52*). The evasion of degradation is not an intrinsic characteristic of p53 mutation (*54*) and is therefore selected for in cancer cells. However, this suggests that pathways that contribute to increasing the levels of mtp53 could be targeted in order to reduce mtp53 load.

One of the few drugs known to be able to reduce mtp53 load is 17-AAG; a Hsp90 inhibitor(*51*). Hsp90 is a chaperone protein which transiently binds numerous proteins and assists in protein folding. Many types of mtp53 appear to require Hsp90 in order to achieve their conformation and, unlike wtp53, bind Hsp90 very stably (*51*). It appears that this binding protects from degradation and if Hsp90 is inhibited it allows the protein to be released and the protein will then become available to be ubiquitinated by the E3 ligase CHIP (*51*). Additionally Hsp90 inhibition also leads to the degradation of a number of other oncogenic proteins which also appear to be dependent on the chaperone. A number of hsp90 inhibitors are currently in clinical trials this includes the hsp90 inhibitor AUY922 which is in phase II clinical trials for breast and gastric cancer (*204*).

The aim of this study is to identify kinase inhibitors which are capable of reducing the amount of mtp53 within a cancer cell line. This will provide further information as to how high levels of mtp53 are sustained within the cell and also

potentially provide possible novel therapeutic targets. The levels of mtp53 in T47D cells (L194F) will be assessed in this screen by in cell western. The in cell western technique is based on similar methodology as western blotting except protein detection by antibody is achieved in a permabilised fixed cell rather than the proteins being extracted first (for full method see chapter 2). This has been shown to be a sensitive technique which provides for much faster screening than western blotting (*205*).

3.4.1 Results

3.4.1.1 Assay validation

In this screen T47D cells; a human breast cancer cell line (mtp53 -L194F) were screened by in cell western for kinase inhibitors capable of reducing the DO1 (p53 specific antibody) signal. The workflow for this screen is summarised in Fig 3.7. DO1 is an ideal antibody to be used in this type of experiment as it does not detect any non-specific bands (Fig 3.8A). This is important in the in cell western technique as non-specific bands cannot be excluded based on size. T47D cells were chosen as the L194F p53 mutation has been shown to be relatively difficult to destroy by 17-AAG in comparison to other cancer cell lines carrying mtp53 (*51*). It is therefore useful to identify mechanisms by which such p53 can be reduced in cells. Additionally, this mutation has been suggested to confer gain of function both in terms of its ability to repress p63 and p73 and also in terms of other pro-tumorigenic effects (*52, 149, 206, 207*). It has been shown in this cell line that repression of the mtp53 by RNAi leads to apoptosis (*52*). Therefore, identification of a drug that could reduce mtp53 levels would be advantageous.

Whilst T47D is not as responsive to 17-AAG as other cell lines, it is still thought to be capable of leading to some reduction in the levels of mtp53 in this cell line (*51*). As 17-AAG is one of the best described drugs capable of reducing mtp53 it was used as a positive control. As can be seen in Fig 3.8 B-C 17-AAG did lead to a reduction in mtp53 levels in T47D cells in this assay.

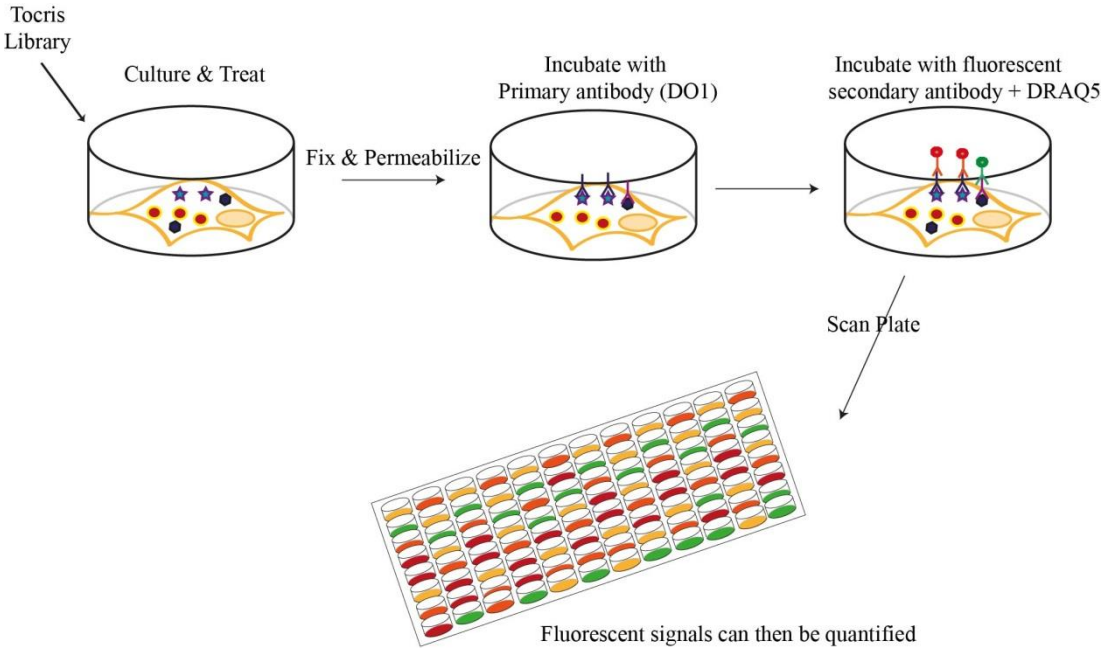

Fig 3.7 Workflow for Screen 3. T47D cells were treated with each compound at 8 different concentrations (0.4µM - 50µM). After 16Hrs cells were fixed and permeabilised. Cells were then incubated with the primary antibody DO1. After washing, the cells were then incubated with the secondary antibody. After a second round of washing the relative amounts of p53 could then be assessed by quantifying the fluorescent signal using the Licor Odyssey scanner. For full details see Materials & Methods.

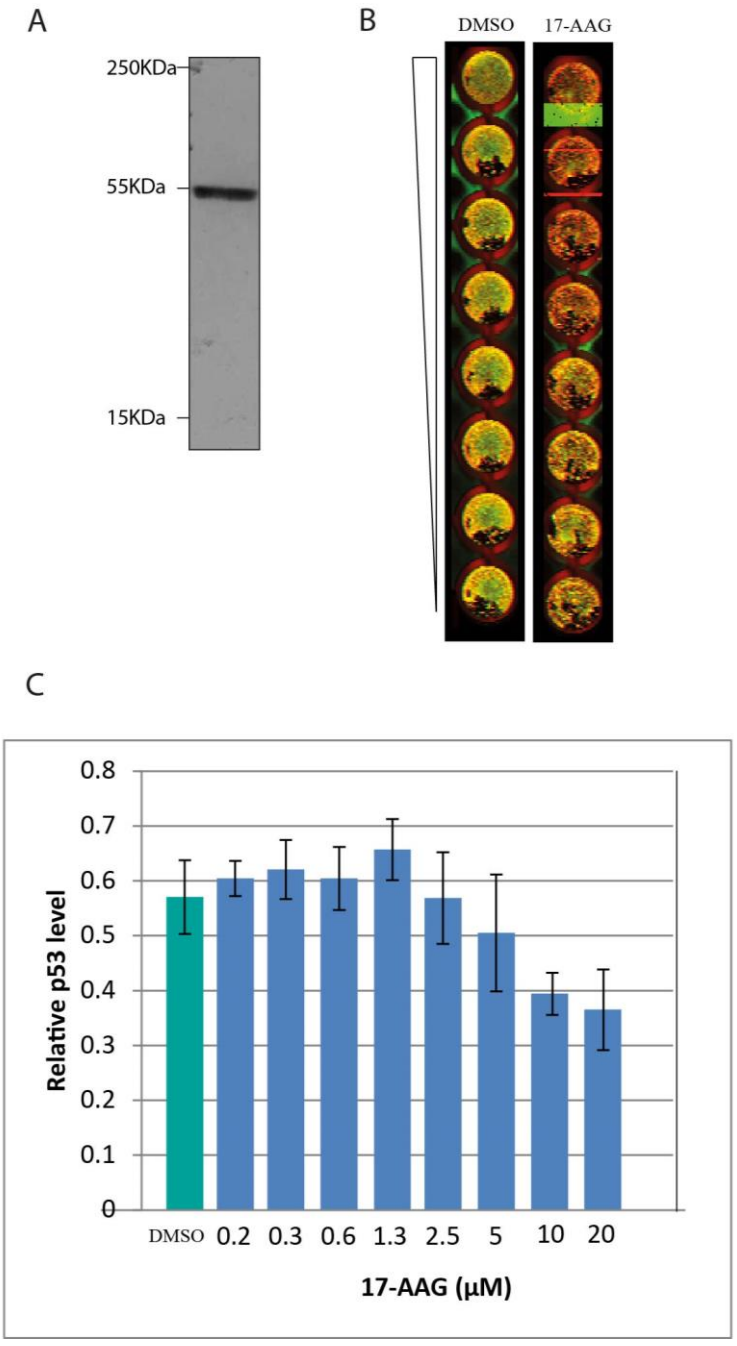

Fig 3.8 In cell western technique can be used to screen for drugs which reduce mtp53 levels. (A) The DO1 antibody used in this assay does not appear to identify non-specific bands. T47D cell lysate was immunoblotted for p53 using DO1. Only 1 band of ~53KDa was observed. **(B)** T47D cells were treated with 17-AAG (20µM-0.2µM) or DMSO for 16Hrs. p53 levels (Green) using the antibody DO1 were assessed by western blotting. Cells were additionally stained with DRAQ5 (red) to assess the relative cell densities. **(C)** T47D cells were treated with the indicated concentration of 17-AAG (or DMSO only) for 16Hrs. p53 levels were assessed by in cell western and normalised to the relative cell densities (determined by DRAQ5 staining).

3.4.1.2 Kinase library screen

The Tocris kinase library was screened, with each drug tested at 8 different concentrations (391nM-50µM). Hits were determined as those where p53 signal appeared to be reduced by comparison to DMSO treated cells and also appeared to have a concentration dependent effect.

Nine kinase inhibitors in the Tocris kinase inhibitor library were identified as potentially reducing total mtp53 protein in this screen and are detailed in Table 3.4. These hits represent six specific targets. Two drugs were both seen to target Cdk, two were seen to target IKKβ and one kinase inhibitor is considered to be a broad spectrum inhibitor. Interestingly 3 of the inhibitors were noted during Screen 1; Aminopurvanol A and IKK16 were noted to cause a loss of cells at higher concentrations and Ro 31-8220 mesylate was noted to cause p53 activation at lower concentrations (0.4µM - 1.6µM) in the wtp53 cells.

Table 3.4. Small molecules identified as reducing mtp53 levels in T47D cells assessed by in cell western

Drug	Target
Rottlerin	PKC
Ro-mesylate	Broad spectrum
Aminopurvanol A	Cdk
Ryuvidine	Cdk
IKK16	IKKβ
IMD0354	IKKβ
PP1	Src
(-)-Terreric acid	BTK
BI 78D3	JNK

3.4.1.3 Preliminary validation

The 9 compounds identified in this screen are potentially capable of reducing mtp53 levels however selection of hits was qualitative. Whilst this screen cannot definitively identify compounds that do or do not lead to alterations in the levels of p53 it provides a means of lead generation. Two of the identified drugs IMD0354 and IKK16 were selected for further analysis. Results of the initial screen were repeated in triplicate in both T47D cells and BT-549 cells. BT-549 cells also have mutant p53 (R249S) so this meant it was possible to determine if the effect of IKK16 and IMD0354 were specific to only T47D cells or the L194F mutant p53. As can be seen in Fig 3.9 IMD0354 leads to a reduction in mtp53 levels in both T47D and BT-549 cells. IKK16 appeared to lead to the reduction in mtp53 in both cell lines but also caused substantial loss of cells from the wells, presumably as a result of cell death, and thus any reduction in mtp53 was difficult to determine. Calculation of p53 intensity relative to cell density (DRAQ5) with higher concentrations of IKK16 lead to negative values. This appeared to result from errors in the determination of total cells (DNA stain) with very low numbers of cells although such errors were not specifically analysed.

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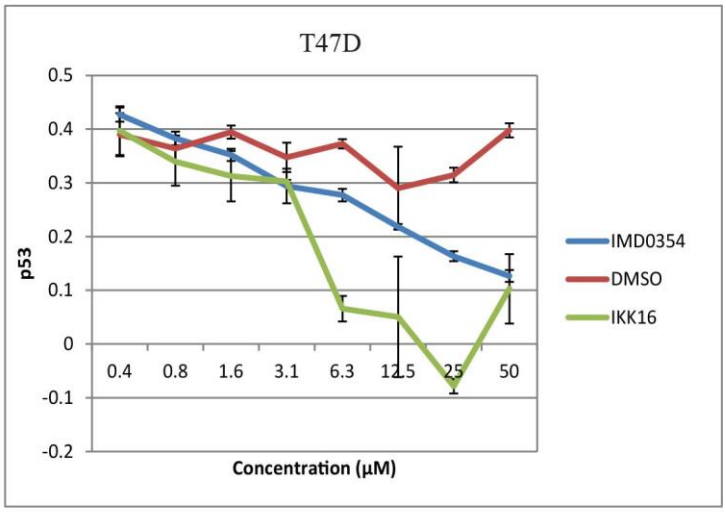

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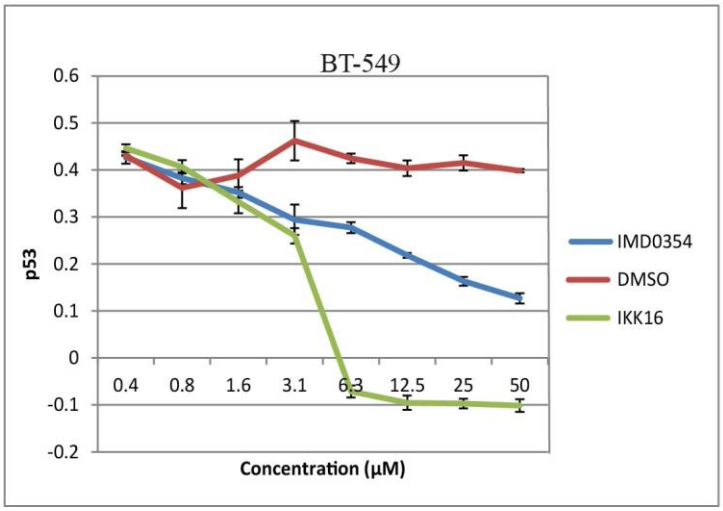


Fig 3.9. IKK16 and IMD0354 reduce p53 levels in T47D and BT-549 cells (A) T47D and **(B)** BT549 cells were treated with the indicated concentration of IMD0354 and IKK16 or the equivalent quantity of DMSO for 16Hrs. Relative p53 levels and relative cell densities were analysed by in cell western using the p53 antibody DO1 and DRAQ5 respectively.

3.4.2 Discussion

In this screen, T47D cells with the p53 mutation L194F were selected for screening. Mutation in p53 at codon 194 is present in roughly 0.5% of cancers with p53 mutation (The p53 Database: http://p53.free.fr/index.html). However, many other mutations found in cancer are also gain of function including some of the most common mutations e.g. 175 present in 5% of cancers with p53 mutation (*41, 43*). The exact number of p53 mutations with gain of function is unknown as the mechanisms for these gains of function are numerous and only just beginning to be understood. Additionally a large number of different mutations have been identified; over 90% of residues have been seen to be mutated in one or more case of cancer (see Fig 1.3).

This screen led to the identification of a number of inhibitors which appeared to reduce levels of mtp53. Further work is required to validate these results but the screen successfully provided a means of lead identification. One interesting target lead is Cdk, this screen identified two Cdk inhibitors which appeared to reduce the levels of mtp53; Aminopurvanol A and Ryuvidine. Curiously, three Cdk inhibitors were identified as being able to activate wt p53 in Screen 1 (Table 3.1). Cdks are a small family of kinases which by definition need to be in complex with a cyclin family protein to be active. Cdks have been shown to have roles in the cell cycle as well as regulation of transcription (*208*). Cdks display specificity towards which cyclins they bind. As the amount of each cyclin protein fluxes during the cell cycle this can control the activity of each Cdk and thus control progression of the cell cycle (*208*). Cdk members are important in control of the cell cycle and some Cdk inhibitors lead to p53 activation and cell cycle arrest (*171*). However some Cdks have been shown to have roles in transcriptional regulation (*209*) and therefore inhibition of some cdks can reduce transcription (*210*) which could provide a mechanism by which p53 is being reduced. The ability of Cdk inhibitors to reduce transcription may be part of the therapeutic value of some such inhibitors (*210*). Cancers are particularly sensitive to such inhibitors, possibly as they are thought to be particularly reliant on rapidly turned over oncogenic proteins.

Chemical inhibitors of Cdks typically show activity towards a number of cdks but each may affect a different subset of the Cdk family. For this reason it would not be expected that all, or even most, cdk inhibitors should produce the same effect. Interestingly of the four cdk inhibitors tested in both assays, two were active in each but none were active in both.

As mentioned, further work will be needed to confirm the ability of these identified inhibitors to reduce mtp53 and further to explore whether the observed effects are due to on-target inhibition. Two of the identified inhibitors targeting IKKβ were however selected for preliminary validation. Repetition of in cell westerns in triplicate confirmed that IMD0354 and IKK16 reduced the DO1 signal in both T47D and BT-549 cells. IKKβ is known to be involved in many processes including regulation of cell cycle, apoptosis, inflammation and immunity (*211, 212*). It has numerous substrates, the best known of which is the transcription factor Nf-κB. There are numerous examples of cross talk between the IKK/Nf-κB and p53 pathways (*39*). For example mtp53 has been shown to enhance Nf-κB transcription(*41*). However it has not been reported, to our knowledge, that IKK sustains mtp53 levels and thus may be an interesting line of inquiry. The interactions between the two pathways will be more fully discussed in the next chapter.

In chapter 4 the effect of IKKβ inhibition on p53 and its pathway will be validated and further defined. Whilst the other inhibitors will not be pursued within the confines of this study this screen has provided a number of compounds potentially capable of effecting mtp53 stability which could be pursued in further studies.

3.5 Conclusion

The above screens identified a number of small molecules that are able to activate wtp53 and reduce the levels of mtp53. No matrices were identified that could activate or repress wtp53. This highlights the usefulness of small molecules in manipulating the p53 pathway. The Tocris kinase inhibitor library was analysed in both Screen 1 and Screen 3. These two screens which aimed to identify compounds

activating wtp53 and reducing mtp53 identified mainly different inhibitors. However, one inhibitor Ro 31-8220 was seen to both activate wtp53 and reduce mtp53, although at different concentration ranges. Ro 31-8220 has previously been seen to activate wtp53 (*213*) but to our knowledge not to reduce mtp53. Ro 31-8220 was first described as a PKC inhibitor (*214*) and interestingly other PKC inhibitors were identified in both screens. GF109203X and Rottlerin were identified in Screen 1 and Screen 3 respectively. As previously discussed PKC has many isoforms which have differential effects on p53 (*175*). Different drugs (or concentrations) may hit different subsets of the PKC proteins and so could lead to opposing effects. An additional problem is that a number of PKC inhibitors have been shown to have off-target effects. For example both Ro 31-8220 and Rottlerin have been shown to inhibit other kinases (*215*). In terms of the effect of Ro 3108220 it is possible that p53 activation at lower concentrations is due to the effect on certain kinases and that at higher concentrations of the drug, which reduce mtp53, additional targets are inhibited.

Another target that was identified in both studies was Cdk, although no specific Cdk inhibitor was identified in both. Again cdks are a family of kinases and so drugs targeting cdk will inhibit a different subset of the family and therefore can lead to different effects. This highlights the importance of characterising individual inhibitors.

In conclusion, these screens have demonstrated that small scale screening is an effective method of identifying potentially novel and dominant pathways in the regulation of wtp53 and mtp53. The screens presented here identified lists of potential p53 regulators; they do not however have the power to confirm whether the these compounds or target molecules are true regulators of p53. Some validation with the data sets can be found, for example, when known regulators are identified this enhances the confidence within the screen. Such screens can provide a useful starting point in the further exploration of regulatory proteins important within the pathway. This project further focuses on two kinase inhibitors (both targeting IKKβ) identified with screen 3 as regulators of mtp53, however future work

could validate and further explore other inhibitor and targets identified in the screens presented within this chapter.

Chapter 4

Inhibition of IKKβ leads to a loss of p53 pathway proteins

4. Inhibition of IKKβ leads to a loss of p53 pathway proteins

4.1 Introduction

p53 is frequently mutated in cancers and this is often associated with accumulation of p53 within the cell (*54, 202, 203*). Despite the high association between p53 mutation and accumulation it has recently been demonstrated that this accumulation is not a condition innately confirmed by the mutation (*54*). As p53 mutation also frequently leads to oncogenic gain of function (*22*), this accumulation is thought to be selected for in cancer cells. Therefore other systems have to be altered within the cell to bring about such accumulation. The implication of this is that these other systems could be targeted in order to reduce the levels of mutant p53 (mtp53).

The final screen in Chapter 3 showed that inhibition of a number of kinases were potentially able to affect the levels of mtp53. This screen was designed to identify kinases potentially involved in mtp53 stability, however further validation is required to show whether these proteins are in fact important in p53 stability. In chapter 3 it was shown that IKKβ inhibitors; IKK16 and IMD0354, led to a reduction in mtp53 signal in in cell westerns. IKK is a multi-subunit complex composed of IKKα, IKKβ and nemo. α and β are kinase subunits whereas nemo is a regulatory subunit with no known kinase activity. IKK is important in many different roles including cell cycle and apoptosis; functions where p53 also have a critical role (*216*). Its contribution to apoptosis is complex and can have both pro and anti-apoptotic roles (*40*). IKK phosphorylates a number of different substrates to mediate these effects but the best known and understood is Nf-κB. Activation of Nf-κB by IKK occurs via two distinct pathways (Fig 4.1)(*212*). In the classic pathway homo or heterodimers of IKKα or IKKβ bound to nemo phosphorylate IkB, which triggers ubiquitination and ultimately degradation of this protein. IkB is a negative regulator of Nf-κB and so active IKK relieves the inhibition of Nf-κB, this allows Nf-κB to enter the nucleus and function as a transcription factor (*212*). In the non-classical pathway homodimers of IKKα directly phosphorylate inhibitory subunits of Nf-κB again allowing active Nf-κB to enter the nucleus (*212*).

The identification of IKK16 and IMD0354 as being potentially capable of modifying mtp53 suggests IKKβ may play a role in mtp53 regulation. Previous research has largely focussed on the effect IKKβ wtp53. Evidence from the literature however shows that there is significant cross-talk between wild type p53(wtp53) and IKK (*39*). Cross-talk between these two pathways is complex and certain studies have shown that IKK acts to oppose the apoptotic effect of p53 whilst others have shown that IKK or the downstream factor, Nf-κB, is required for p53 mediated apoptosis.

IKK/Nf-κB have, however, largely been thought of as anti-apoptotic factors. This is supported by the fact that the pathway is hyper-activated in a large number of cancers and appears to contribute to survival (*99*). The IKK and p53 pathways have been shown to be oppositely regulated by a number of pathways. For example, the Akt pathway has been shown to repress p53 by positive regulation of MDM2 (*217*) and also phosphorylates IKK which leads to the activation of Nf-κB (*218*). On the other hand ARF represses MDM2 activity leading to increased p53 activity (*88*) whilst repressing Nf-κB by inhibition of the Nf-κB subunit Rel A (*219*).

It has also been shown that the two pathways can directly oppose each other. For example, Nf-κB has been shown to lead to positive regulation of MDM2 (*220-222*) which would inhibit p53 activity. A number of mechanisms by which this can happen have been demonstrated although the factors controlling which mechanism is observed are unknown. In MEFs Nf-κB has been shown to lead to the up-regulation of MDM2 mRNA transcription via a mechanism which is dependent on both p53 and Nf-κB but which does not result from direct binding of Nf-κB to the promoter (*220*). Busuttil et al. (2010) however showed that in T-cells Nf-κB could lead to the direct increase in MDM2 mRNA via binding of Nf-κB to the P1 promoter (*221*). Although within the context of T-cells this lead to p73 mediated apoptosis rather than p53 mediated effects. Lau et al. (2012) also recently showed that IKK can also lead to the post-translational modification of MDM2 as well as transcriptional changes (*222*). They demonstrated that Nf-κB pathway activation by knockdown of the Nf-κB pathway inhibitor cIAP2 leads to increased MDM2 activity; this resulted

from a transient increase in mRNA levels and a sustained IKK dependent increase in phosphorylation and sumolyation of MDM2. This resulted in decreased p53 levels.

IKK can also inhibit the p53 pathway via Nf-κB independent mechanisms. IKKβ can directly phosphorylate p53 at ser362 and ser366 which leads to its degradation via the E3 ligase β-TrCP1 (*223*). Furthermore IKKβ can also indirectly inhibit p53 transcriptional activities. IKKβ has been shown to phosphorylate ΔNp73α at ser422 (*224*). ΔNp73α is an isoform of the p53 family protein p73 and it has been shown to repress the activity of both p53 and p73 (*72*). When ΔNp73α is phosphorylated by IKKβ its ability to bind response elements of p53 controlled genes is enhanced and thus prevents the binding and transcriptional activities of p53 (*224*).

However, as mentioned previously, IKK is not just an anti-apoptotic factor and in certain situations can be pro-apoptotic. In some cases IKK has also been seen to promote p53 activity/stability. Fujioka et al. (2004) showed that the superoxide inducing agent doxycycline leads to the activation of both Nf-κB and p53 and that the activation of p53 was dependent on Nf-κB (*225*). The authors showed that Nf-κB induced p53 activity by both transcriptional and post-translational mechanisms. They showed that p53 mRNA levels were increased, MDM2 levels were reduced and phosphorylation of p53 at ser20 was increased; demonstrating that Nf-kB can regulate p53 activity at multiple levels. IKK has also been shown to positively regulate the p53 pathway via Nf-κB independent means. During H_2O_2 stress IKKβ has been shown to phosphorylate and activate S6K1 which leads to the phosphorylation of MDM2 at Ser166 (*226*). Phosphorylation at this residue leads to inhibition of MDM2 nuclear entry meaning that it is unable to promote nuclear p53 degradation (*226*). This pathway was shown to be important in mediating cell death resulting from H_2O_2 stress (*226*).

The mechanisms controlling when or how IKK activation is pro or anti-apoptotic are not well understood, although progress is being made in this area. It is known that the outcome of IKK/Nf-κB activation appears to be at least in part dependent on the initial stimuli: Lin et al. (1999) showed that whilst both T-cell

activators and glucocorticoids can both induce apoptosis within T-cells, ablation of Nf-κB activity has opposite effects (*40*). Blockade of Nf-κB activity enhanced glucocorticoid mediated apoptosis but conversely T-cell activation induced cell death by PMA/ionomycin appears to require Nf-κB and its inhibition reduced apoptosis. Ryan et al. (2002) showed that induction of p53 activity lead to an increase in Nf-κB which was required for cell death (*227*). Conversely they found that Nf-κB acted to protect cells in TNFα induced cell death. The authors suggested that activation of Nf-κB in these systems relied on different mechanisms. They showed that NIK, an upstream mediator of IKK, was required for TNFα induced, but not p53 induced, Nf-κB. Activation of Nf-κB by p53 was instead reliant on MEK and $pp90^{rsk}$. Consistent with this result O'Prey et al. (2010) also showed that TNFα leads to Nf-κB acting as an anti-apoptotic factor but p53 activation required Nf-κB for an apoptotic response (*228*). They further elucidate this mechanism by showing that Nf-κB was not required for the regulation of all down-stream p53 targets but was required for the pro-apoptotic factors, p53AIP1 and Noxa.

The impact of this cross-talk between IKK and p53 in cells with mtp53 is unclear. As discussed, IKK can act to promote or inhibit p53 activity and/or stability and the outcome is, at least partly, determined by stimulus. Clearly some of the regulatory mechanisms discussed would likely not have the same impact on mtp53 as wtp53. For example, MDM2 is poorly able to modulate mtp53 levels (*50*) and thus regulation via this route may be reduced. Furthermore the IKK/Nf-kB pathway is often deranged in cancer further complicating our understanding of the interaction between these two pathways in cancer. In fact it has been shown that hyper-activation of the IKK pathway may be associated with mutation of p53 (*42*). Mtp53 is usually highly stable in terms of its half-life in cancer (*202, 203*) suggesting that it may not be significantly downregulated by the hyperactive IKK pathway. Empirical evidence shows that certain mutants of p53 are able to up-regulate the Nf-κB pathway. For example, mtp53 (R175H, R273H, and D281G) appears to act to promote Nf-κB via increased transcription and thus enhance its anti-apoptotic abilities (*41*). This provides a potential mechanism for the association of mutation of

p53 and IKK pathway activation. However, if inhibition of IKK is confirmed to reduce mtp53 levels this suggests that IKK could also act to increase mtp53 stability. The following chapter examines the potential for small molecule inhibitors of IKKβ (IMD0354/IKK16) to alter mtp53 and wtp53 in cancer cells. Second, it seeks to identify whether other proteins within the p53 pathway (specifically MDM2 and p21) are altered by IMD0354 treatment. Finally, it attempts to address the mechanisms by which this change is occurring.

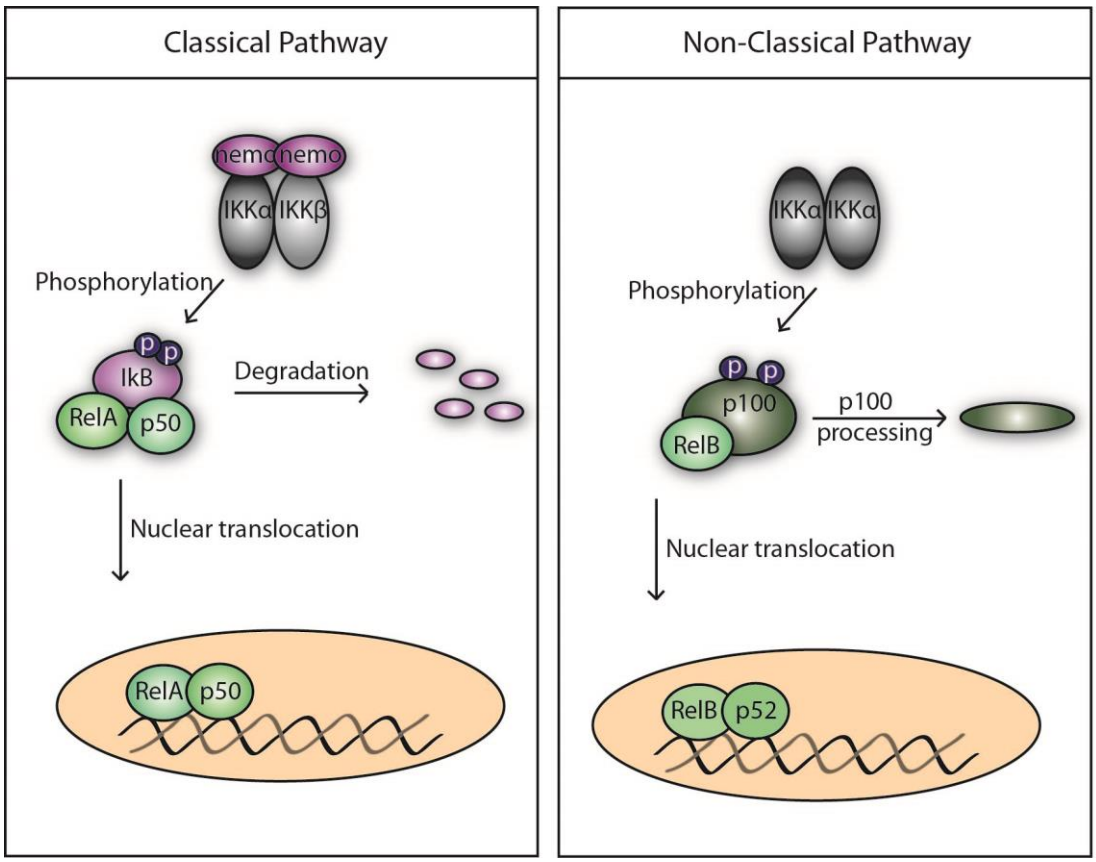


Fig 4.1. Nf-κB can be activated by two IKK dependent pathways. (A) In the Classical pathway upon activation of the IKK complex IkB is phosphorylated. This causes IkB to dissociate from Nf-κB subunits RelA and p50 and be targeted for degradation. This exposes the nuclear localisation sequence on Nf-κB subunits allowing them to enter the nucleus. **(B)** In the Non-Classical pathway activated IKKα dimers phosphorylate p100. This both allows RelB to be released and also promotes the processing of p100 to the active p52 form. Both RelB and p52 can then enter the nucleus.

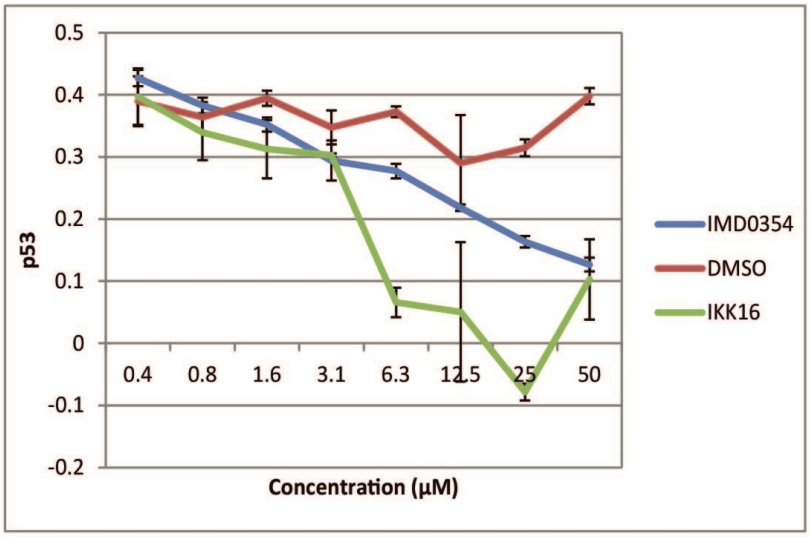
4.2 Results

4.2.1 mtp53 is reduced by the IKKβ inhibitor IMD0354

The final screen (Screen 3) in Chapter 3 revealed a number of small molecule inhibitors which appeared to reduce the levels of mtp53 (L194F) in T47D breast cancer cells as shown by the in cell western technique. This included the IKKβ inhibitor, IMD0354, and the mechanism by which this drug affects the p53 pathway will be explored in this chapter. A second IKKβ inhibitor, IKK16 was also identified to reduce mtp53 in the same screen; however, this inhibitor caused significant cell death, so therefore further work focuses mainly on IMD0354. IMD0354 has been proposed as a potentially useful chemotherapeutic and has been shown to have anti-cancer effects in mice xenograph experiments (*229, 230*). It is therefore important to fully understand the action of this drug.

In cell westerns reflect the total epitope availability within a cell and with a highly specific antibody such as DO1 (used in this study) all the p53 within the cell can be quantified. However, any technique may have a degree of unspecificity and therefore it is advantageous to confirm the results by another technique. Western blotting was employed to confirm the reduction of p53 by IMD0354 as this is a long standing reliable technique. Fig 4.2 shows the results of this, as well as in cell western results for comparison. As can be seen in Fig. 4.2 western blotting replicates the reduction of p53 levels in T47D cells after IMD0354 treatment and shows a reduction in mtp53 levels over a similar concentration range (~6.3-50μM). This confirms that treatment of T47D cells with IMD0354 does lead to a reduction in mtp53.

A



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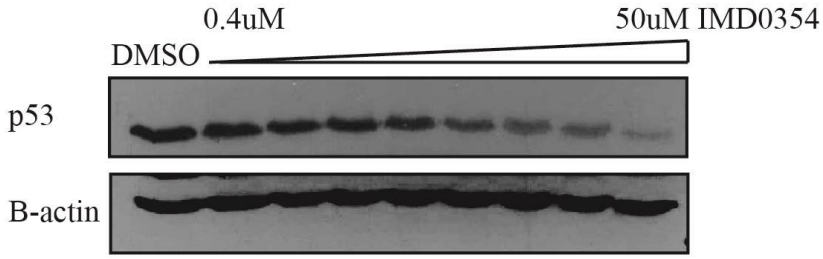


Fig 4.2. IMD0354 treatment leads to a reduction in mtp53 levels in T47D. (A) T47D cells were treated with IMD0354 at the indicated concentration for 16Hrs. p53 levels were then assessed by in cell western. The levels of p53 were assessed using the p53 specific antibody DO1 and were normalised to DRAQ5 levels to control for cell number variation. Values of p53 levels are mean values from results in triplicate from a single plate. Error bars show standard deviation. **(B)** T47D cells were treated with IMD0354 at the indicated concentration for 16Hrs. Cell lysates (20µg/well) were then assessed by western blotting to determine relative levels of p53 by western blotting using anti-p53 (DO1). Equal loading was also assessed using anti-β-actin antibody.

4.2.2 Wild-type p53 is also reduced by IMD0354.

p53 is a tightly regulated protein which is affected by a number of systems (*231*). Mutation in p53 often leads it to escape from some normal forms of regulation but still retain other forms (*50*). Mutation in p53 may also cause it to become differently regulated by certain mechanisms in comparison to the wild type form (*51*). It is therefore important to know whether the inhibition of IKKβ will affect wtp53 in the same way as it affects mutant p53.

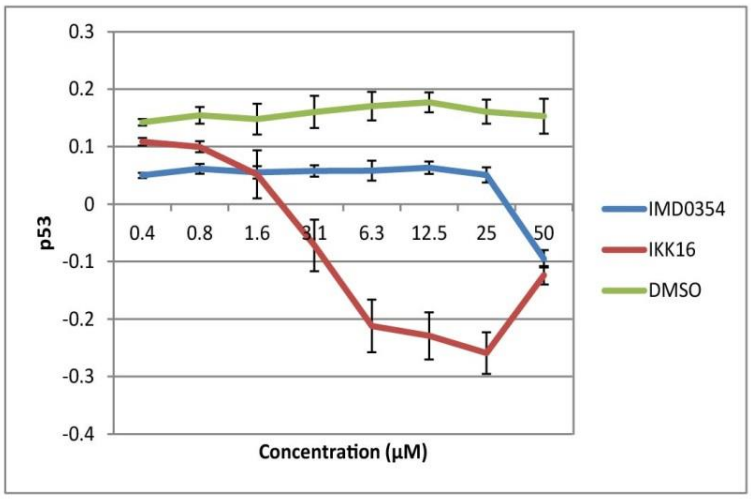
As shown in Fig. 4.3A treatment of A375 cells (wtp53) also leads to a reduction in the levels of p53 as observed using in cell western. Once again, following this result it was sought to confirm whether such an effect could be observed using western blotting. As seen in Fig 4.3B western blotting showed that wtp53 in A375 cells was generally reduced by IMD0354 treatment, however, fluctuations were seen in the levels of p53 across the concentration range. Whilst a reduction in wtp53 was seen using both techniques minor differences in the levels of p53 at each concentration were observed and it should be noted that a strict concentration dependent effect was not seen.

Western blotting showed that IMD0354 caused small fluctuations in the expected steady decrease in p53 being observed. Fluctuations in p53 pathway proteins are often observed as a result of regulatory feedback mechanisms (*164*). Such fluctuations were not seen in mtp53 cells, this is perhaps unsurprising as mtp53 does not induce MDM2 and so is not subject to the same regulatory feedback mechanisms (*163*). Furthermore, mtp53 frequently accumulates as it escapes normal degradation and thus has significantly enhanced stability (*50*). As the level of wtp53 will, to a relatively small degree, be affected by where in the fluctuating cycle p53 is when the snapshot is taken this may explain why some differences are observed in the effects seen by western blotting and in cell western.

As discussed whilst the results from the two techniques showed a similar trend they were not identical. Result from such different techniques are likely to vary as in cell westerns will detect p53 in the cell, which has an available DO1 epitope after cell permeabilisation, whereas western blotting relies on a lysis buffer

to extract p53 prior to detection. Therefore, there may be differences in the pools of p53 available in the two techniques. As western blotting is the more fully defined and established technique further work will mainly focus on this method.

A



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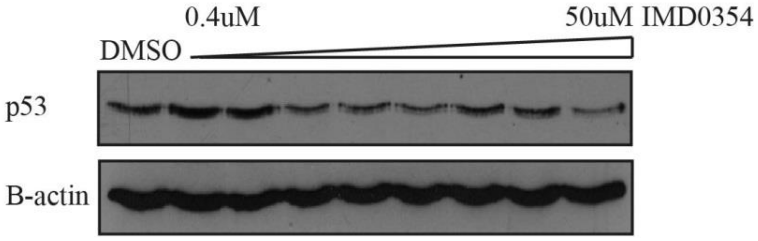
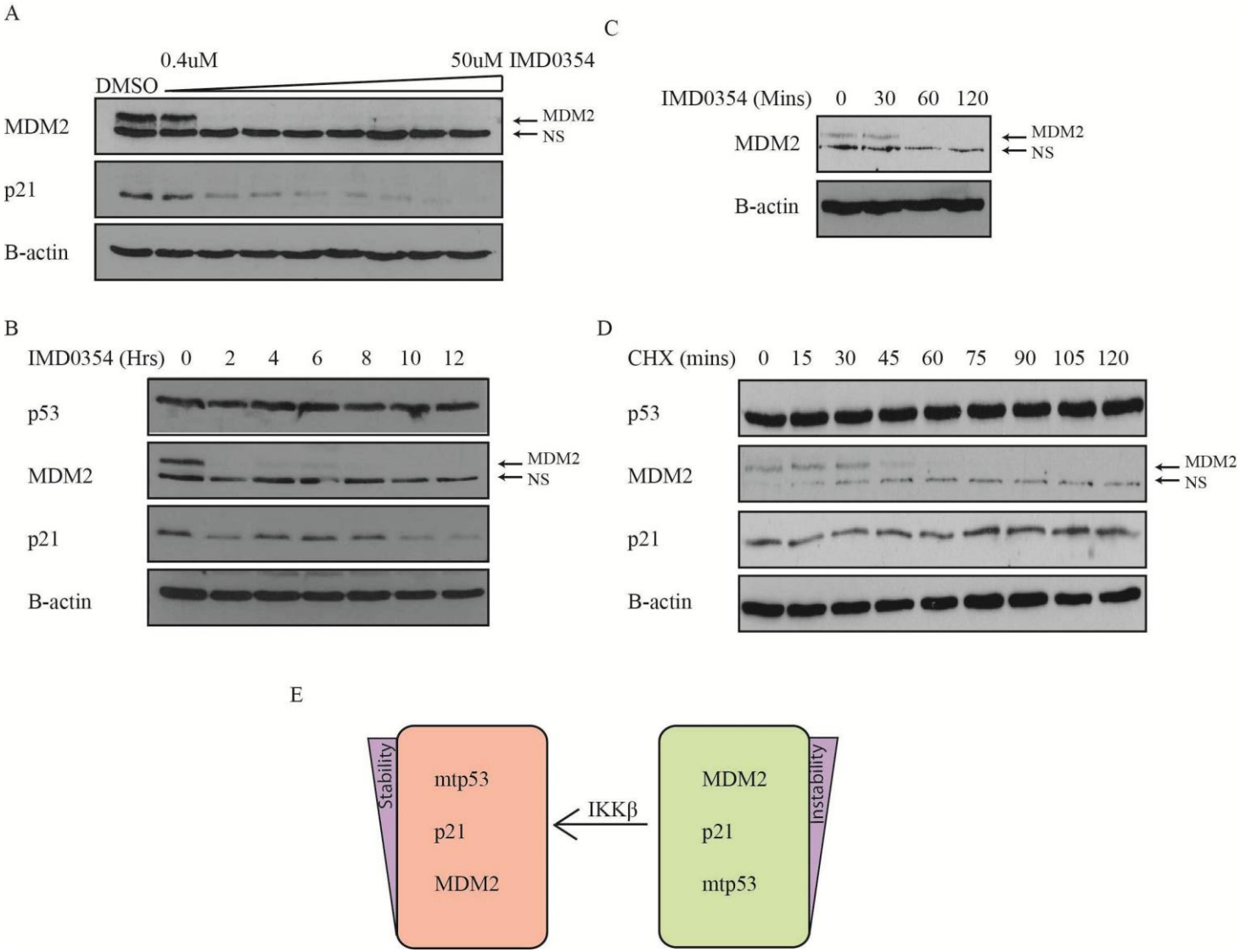


Fig 4.3. IMD0354 treatment leads to a reduction in mtp53 levels in A375. (A) A375 cells were treated with IMD0354 at the indicated concentration for 16Hrs. p53 levels were then assessed by in cell western. The levels of p53 were assessed using the p53 specific antibody DO1 and were normalised to DRAQ5 levels to control for cell number variation. Values of p53 levels are mean values from results in triplicate from a single plate. Error bars show standard deviation. **(B)** A375 cells were treated with IMD0354 at the indicated concentration for 16Hrs. Cell lysates (20µg/well) were then assessed by western blotting to determine relative levels of p53 using anti-p53 (DO1). Equal loading was also assessed using anti-β-actin antibody.

4.2.3 IMD0354 leads to a reduction in the levels of p53 pathway proteins.

The stability and activity of p53 is often best considered in the context of its pathway proteins. Whilst the p53 in T47D cells is mutated it can still be subject to some, although more limited, alteration by its pathway proteins, for example whilst MDM2 poorly ubiquitinates p53, it can still play some, albeit more limited, role in mtp53 degradation (*50*). Additionally some mtp53 has been shown to be able to cause the basal transcription of some of its normal responsive genes (*37*). For these reasons the levels of the p53 pathway proteins MDM2 and p21 were investigated when T47D cells were treated with IMD0354. As can be seen in Fig. 4.4 both MDM2 and p21 appear to be reduced by IMD0354 treatment. As can be seen in Fig. 4.4 B, C MDM2 is the most acutely affected by IMD0354 with p21 affected second and p53 sometime after that. The order of loss of the p53 pathway proteins appears to reflect the relative stability of these proteins within the cell (Fig 4.4D). As can be seen in Fig 4.4 MDM2, which is lost first, has the fastest turnover.

Subsequent work focuses on investigating the effect of IKKβ on MDM2 as it is the most rapidly and acutely affected p53 pathway protein identified. Furthermore MDM2 is also considered to be an oncogenic protein and is overexpressed in a number of cancers (*26*). It appears to promote oncogenicity both via its inhibition of p53 but also via a number of p53 independent mechanisms (*232*). Thus reduction of MDM2 protein levels or activity may be advantageous. Inhibition of MDM2 activity by Nutlin, which was specially designed to inhibit MDM2 repression of p53, is thought to be beneficial in cells with wild-type p53 (*108*). However some studies also suggest that Nutlin can also have p53 independent anti-tumourigenic effects (*233*). Knock-down of MDM2 has revealed that reduction of MDM2 can, at least in certain cells, lead to p53-independent reduced cell growth and apoptosis (*234, 235*).


A
0.4uM
50uM IMD0354
DMSO
MDM2
p21
B-actin
MDM2
NS
C
IMD0354 (Mins) 0 30 60 120
MDM2
B-actin
MDM2
NS
B
IMD0354 (Hrs) 0 2 4 6 8 10 12
p53
MDM2
p21
B-actin
MDM2
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D
CHX (mins) 0 15 30 45 60 75 90 105 120
p53
MDM2
p21
B-actin
MDM2
NS
E
Stability
mtp53
p21
MDM2
IKKβ
MDM2
p21
mtp53
Instability

Fig 4.4 IMD0354 leads to a reduction in p53 pathway proteins; MDM2 and p21. (A) T47D cells were treated with IMD0354 at the indicated concentration for 16Hrs. Cell lysates (20µg/well) were then by western blotting assessed to determine relative MDM2 and p21 levels by western blotting using anti-MDM2 (2A10) and anti-p21 antibodies. Equal loading was also assessed using anti-β-actin antibody. **(B)** T47D cells were treated with IMD0354 for the indicated time. Cell lysates (20µg/well) were then assessed by western blotting to determine relative p53, MDM2 and p21 levels by western blotting using anti-p53 (DO1), anti-MDM2 (2A10) and anti-p21 antibodies. Equal loading was also assessed using anti-β-actin antibody. **(C)** T47D cells were treated with 5µM IMD0354 for the indicated time. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody. **(D)** T47D cells were treated with 10µM cycloheximide (CHX) for the indicated time. Cell lysates (20µg/well) were then assessed by western blotting to determine relative p53, MDM2 and p21 levels by western blotting using anti-p53 (DO1), anti-MDM2 (2A10) and anti-p21 antibodies. Equal loading was also assessed using anti-β-actin antibody. **(E)** Schematic showing the model that IKKβ enhances the stability of p53, MDM2 and p21, and that when inhibited the observed order of loss will depend on the proteins turnover rate.

4.2.4 IMD0354 leads to a reduction in MDM2 protein.

Western blotting using the MDM2 specific antibody, 2A10 (Fig 4.4 A-D) showed that MDM2 appear to be reduced by IMD0354 treatment. However, several bands are seen with 2A10 and this result is based on the assumption that MDM2 is represented by the higher band of ~90KDa. This is consistent with the fact that MDM2 is usually expected to run at ~90KDa on an SDS gel. However in order to confirm this, siRNA targeting MDM2 was used. As shown in Fig 4.5A MDM2 siRNA leads to a reduction in the higher 2A10 band confirming that the ~90KDa band does represent a form of MDM2.

2A10 is a widely used and specific antibody however it has been shown that phosphorylation of the protein can mask the epitope (*236*). This suggests that the observed loss of the 2A10 epitope with IMD0354 treatment could be due to either phosphorylation changes or a change in total MDM2. In order to ascertain which is the case a second antibody, 4B2, was used. Whilst 2A10 binds MDM2 at amino acids 390-396, 4B2 binds at a distinct site at amino acids 19-50. As can be seen in Fig. 4.5B-E the reduction in MDM2 occurs at the same concentration of IMD0354 using either 2A10 or 4B2 by western blotting or in cell western. This confirms that IMD0354 is leading to a reduction in total MDM2.

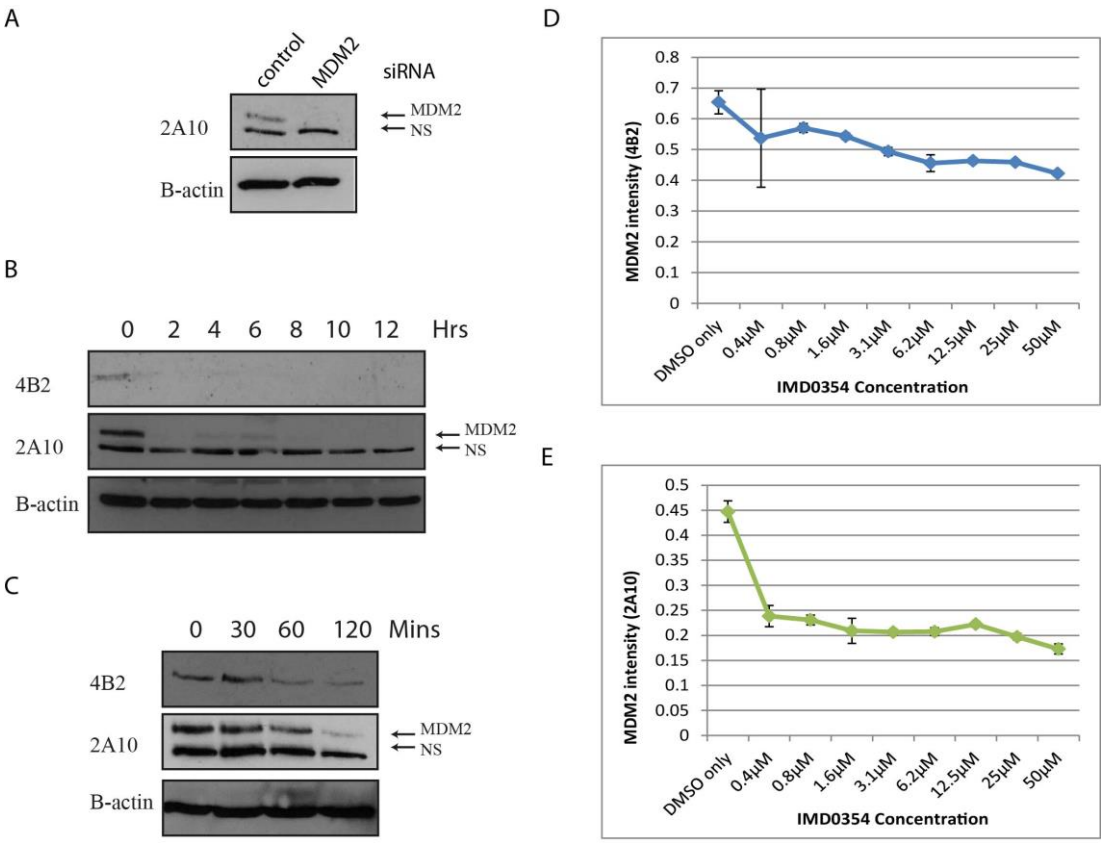

Fig 4.5. Loss of the MDM2 epitope reflects the loss of the MDM2 protein. (A) T47D cells were treated with 40nM control non-targeting siRNA or siRNA targeting MDM2 for 48Hrs. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody. **(B, C)** T47D cells were treated with IMD0354 for the indicated time. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody. **(D,E)** T47D cells were treated with the indicated concentration of IMD0354 for 16Hrs. Levels of MDM2 were then analysed by in cell western using the antibodies 2A10 and 4B2. The relative levels of fluorescence were then quantified using the Licor software. 4B2 **(D)** and 2A10 **(E)** levels were corrected for background (secondary only) fluorescence and normalised to DRAQ5 to control for cell number

4.2.5 The reduction in MDM2 is independent of p53

The level of MDM2 in a cell is regulated by a number of factors. MDM2 protein levels are usually altered as a result of changes to transcription as well as post-translational modification altering degradation rate (*237-239*). Transcription of MDM2 is known to be under the control of p53 and acts in a positive feedback loop by negatively regulating p53 (*239*). Therefore the observed reduction in MDM2 could result from a loss of p53. Mutant p53 frequently losses its ability to transcribe genes normally under its control, however some studies have suggested that the loss of transcriptional activity is not absolute in some p53 mutations (*37*). As the reduction in MDM2 precedes the reduction in p53 this suggests that MDM2 loss is not dependent on p53 in this case. However, activity is not solely dependent on protein level and so a loss of activity of p53 could precede a loss of protein. To test whether the reduction in MDM2 is dependent on p53, the effect of IMD0354 was tested in a number of cell lines with different p53 statuses. A375, MDA-MB-231 and H1299 which have wtp53, mtp53 (R280K) and p53-null respectively were treated with IMD0354 and the effect on MDM2 level was tested by immunoblotting. As can be seen in Fig. 4.6 MDM2 was reduced in all the cells lines tested showing that the presence or absence of functional p53 does not affect the loss of MDM2 caused by IMD0354.

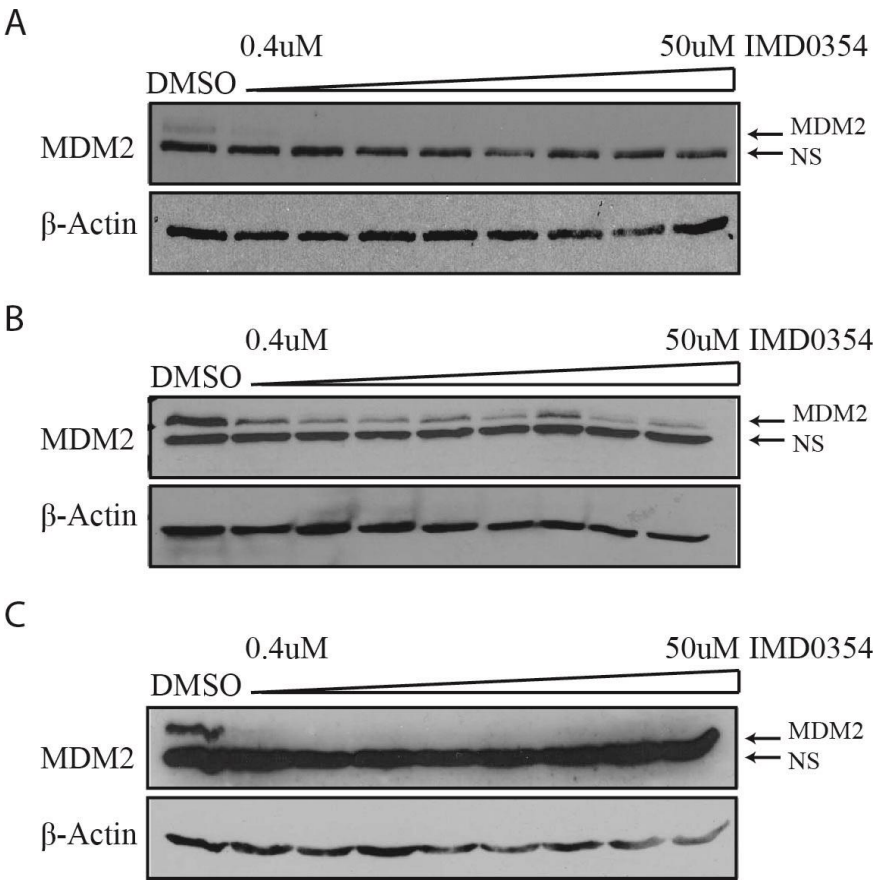

Fig 4.6. The reduction in MDM2 as a result of IKKβ inhibition is not dependent on p53 status. A375 **(A)**, MDA-MB-231 **(B)** and H1299 **(C)** cells were treated with the indicated concentrations of IMD0354 for 16Hrs. Their p53 status is wild-type, mutant (R280K) and null respectively. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody.

4.2.6. IMD0354 inhibits the Nf-κB pathway in T47D cells

Next, we wanted to assess whether IMD0354 effectively inhibits IKKβ. IMD0354 is thought to specifically inhibit IKKβ via inhibition of ATP binding (*240*). Therefore, the effect of IMD0354 on IKKβ can be measured indirectly through its effect on downstream components. Tanaka et al. (2006) investigated the effect of IMD0354 on the cell cycle and showed that a number of changes occurred which lead to cell cycle arrest (*229*). For example, they showed that IMD0354 leads to a reduction in IκB phosphorylation as well as reducing cyclin D1 and D3. Such changes are consistent with the effect of IKK inhibition. IκBα is one of the substrates of IKK (*241, 242*) and therefore will have reduced phosphorylation when IKK is inactive. Reduced phosphorylation would mean that more IκB is bound to Nf-κB keeping it in its inactive state and so the levels of proteins whose gene transcription is promoted by Nf-κB would fall, these genes include D-type cyclins (*243*). To see whether IMD0354 acts in this way, and is therefore hitting its target in T47D cells IκBα, phospho-IκBα, cyclin D1 and cyclin D3 levels were assessed by western blotting in cells treated with IMD0354. Due to the fact phosphorylation of IKBα leads to its rapid degradation cells were also treated with MG132 in order to observe phospho-IKBα. As can be seen from Fig. 4.7 A-B there is a small increase in the levels of IKBα which is concomitant with a decrease in the relative phosphorylation levels after 60mins. Interestingly however levels of phosphorylated IKBα temporarily increase after 30mins before dropping. Additionally levels of cyclin D1 and cyclin D3 were both reduced over time with IMD0354 treatment (Fig 4.7C).

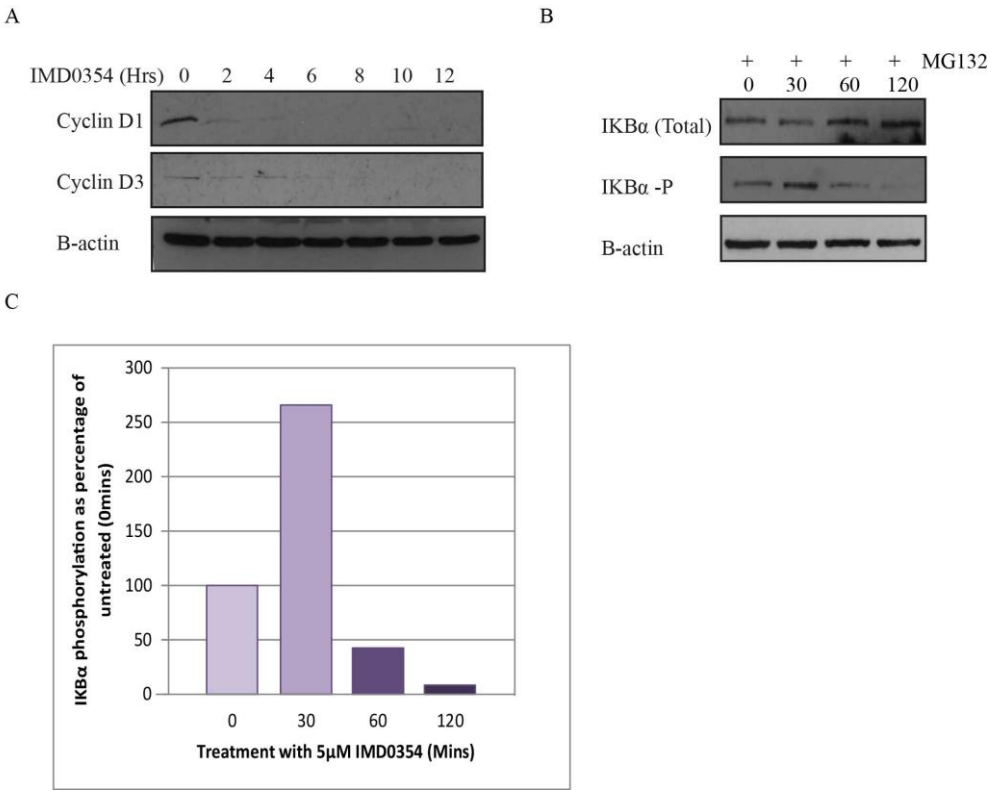

Fig 4.7. IMD0354 leads to a reduction in D-type cyclins and a reduction in IKBα phosphorylation. **(A)** T47D cells were treated with the 50μM IMD0354 for the indicated time. Cell lysates (20μg/well) were then assessed by western blotting to determine relative Cyclin D1 and Cyclin D3 levels using the anti-Cyclin D1 and Cyclin D3 antibodies. Equal loading was also assessed using anti-β-actin antibody. **(B)** T47D cells were treated with the MG132 for 3Hrs in addition to 5μM IMD0354 for 2Hrs, 1Hrs, 30mins or 0mins . Cell lysates (20μg/well) were then assessed by western blotting to determine relative IKBα and phospho-IKBα levels using the anti-IKBα and anti-phospho-IKBα antibodies. Equal loading was also assessed using anti-β-actin antibody. **(C)** The change in phospho-IKBα relative to total IKBα was then quantified using ImageJ.

4.2.7 Inhibition of IKKβ reduces MDM2

The above results demonstrate that IMD0354 leads to a reduction in MDM2 levels as well as reducing the activity of IKKβ over a similar time scale. It is not clear however whether it is the ability to inhibit IKKβ that causes IMD0354 to reduce MDM2 levels. In order to establish whether this ability to reduce MDM2 levels is common to other chemical inhibitors of IKKβ two other IKKβ inhibitors were tested. The inhibitors used were IKK16 and BMS-345541, both of which are considered to be IKKβ specific inhibitors (*244, 245*). BMS-345541 was tested using concentrations ranging from 0.4μM-50μM, IKK16 was only tested from 0.4μM-25μM as, consistent with previous results, higher concentrations caused a high level of cell death. As shown in Fig 4.8. in T47D cells both IKK16 and BMS-345541 lead to a reduction in MDM2 levels. Whilst these three compounds all target IKKβ they are structurally unrelated (Fig 4.9), this suggests the chemical perturbation of IKKβ function consistently leads to a reduction in MDM2.

IKK16 and BMS-345541 were also tested in A375 cells in order to ascertain their effect on wtp53. A375 appeared to be more sensitive to IKK16 than T47D cells and lead to significant cell death; as such no results were obtained for this drug in A375 cells. However, consistent with the results of IMD0354, BMS-345541 also lead to a reduction in MDM2 in A375 cells (Fig 4.10). Of the 3 drugs tested IMD0354 appeared to be the most effective in reducing MDM2 in both cell lines and could reduce MDM2 levels at concentrations as low as ~800nM.

Together, the results of treatment of cells with IMD0354, IKK16 and BMS-345541 demonstrate that chemical inhibition of IKKβ leads to a reduction in MDM2 protein levels which is independent of p53 status. Chemical inhibition generally reduces the kinase activity of its given target. However a number of kinases including the IKK family protein IKKα have been shown to have additional functions independent of their kinase activity (*246*). Any kinase independent function of IKKβ may remain undisrupted by chemicals inhibiting it kinase function but this is not assured. Potentially reducing its kinase function could make the protein more available for other non-kinase functions. In order to confirm that the reduction in

MDM2 results from a reduction of IKKβ function and not as a result of an increase in non –kinase function or, alternatively, off-target effects of the specific inhibitors, siRNA targeting IKKβ was utilised to disrupt IKKβ.

T47D and A375 cells were treated with either siRNA targeting IKKβ or non-targeting siRNA. As can be seen in Fig 4.11 treatment of T47D and A375 cells with IKKβ siRNA leads to a reduction in MDM2 levels compared to treatment with non-targeting siRNA. This reduction appears to be less substantial than with inhibitor treatment. This shows that, at least qualitatively, the effect of chemical and genetic inhibition is the same and confirms that inhibition of IKKβ leads to a reduction in MDM2.

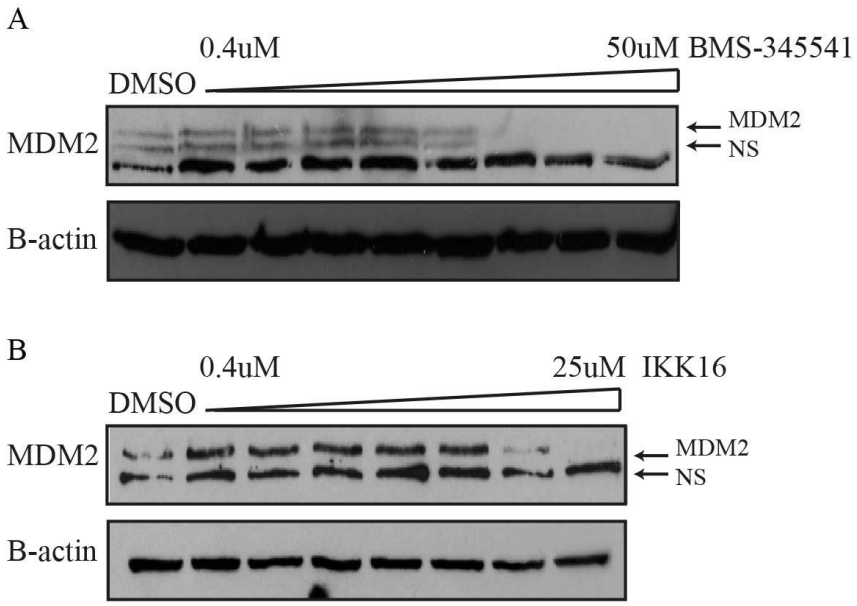

Fig 4.8. IKKβ inhibitors BMS-345541 and IKK16 led to a reduction in MDM2 in T47D cells. T47D cells were treated with the indicated concentration of BMS-345541 **(A)** or IKK16 **(B)** for 16Hrs. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody.

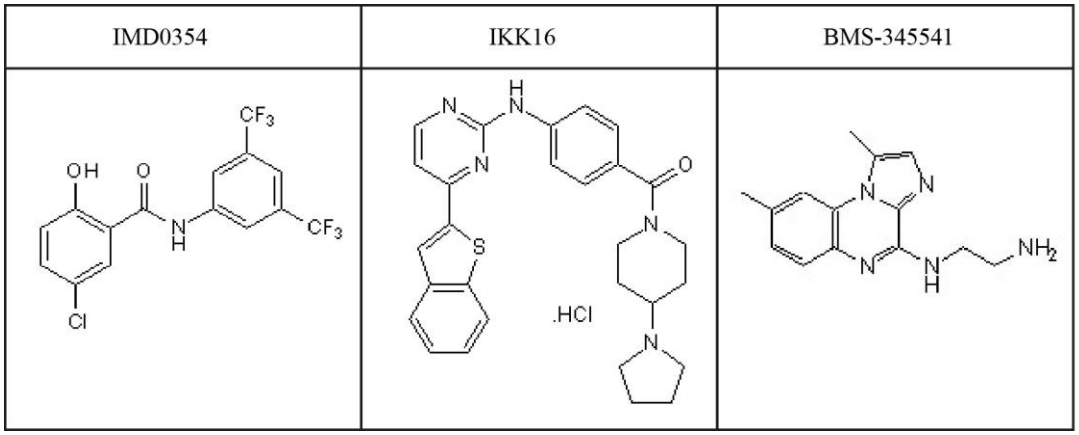

Fig 4.9 Structure of the IKKβ inhibitors IMD0354, IKK16 and BMS-345541

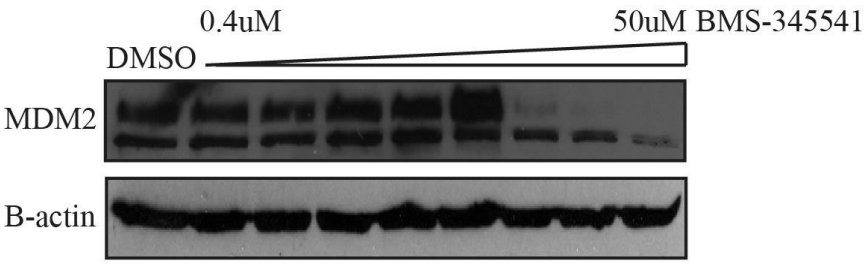

Fig 4.10. IKKβ inhibitor BMS-345541 led to the reduction of MDM2 in A375 cells. A375 cells were treated with the indicated concentration of BMS-345541 for 16Hrs. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody.

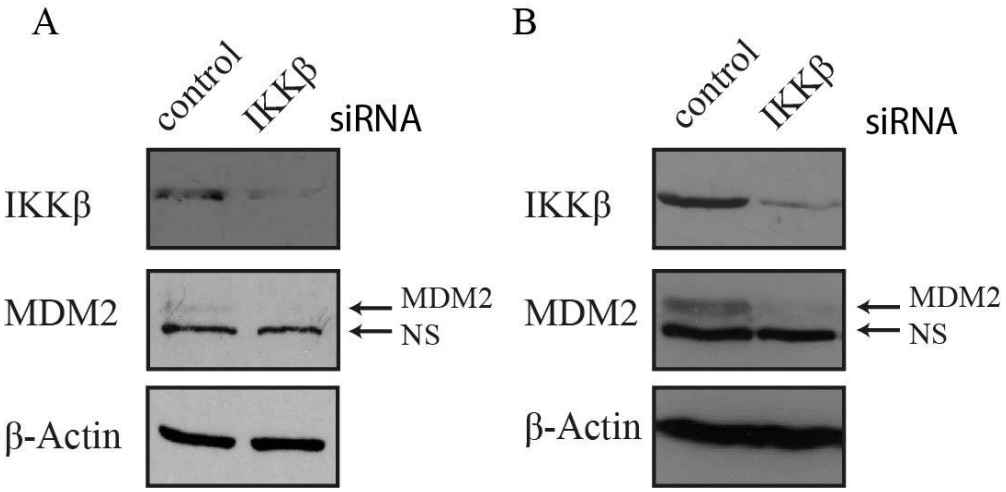

Fig. 4.11. siRNA targeting IKKβ recapitulates the reduction of MDM2 seen with chemical inhibition. T47D **(A)** and A375 **(B)** cells were transfected with control, non-targeting and IKKβ specific siRNA. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 and IKKβ levels using anti-MDM2 and anti-IKKβ antibodies. Equal loading was also assessed using anti-β-actin antibody.

4.2.8 Overexpression IKKβ does not lead to substantial changes in MDM2 levels

This study shows that inhibition of IKKβ by siRNA or chemical inhibition leads to the reduction of MDM2. The next question to be addressed therefore was whether overexpression of IKKβ would similarly lead to an increase in MDM2. Therefore we sought to investigate the effect on MDM2 of the overexpression of wild-type, constitutively active and kinase dead IKKβ. IKKβ is activated when phosphorylated at two serines, 177 and 181, within the T-loop domain (*242, 247*). It has been shown that if these serines are replaced with glutamic acid to mimic the phosphorylated form, the protein becomes constitutively active (*241, 242*). Conversely mutation at the K44 residue within the ATP binding domain can cause reduced kinase activity presumably as a result of poor ATP binding (*241, 242*).

In order to assess the effect of overexpression of the different IKKβ mutants, stable cell lines expressing wild-type, K44M (KM) and S177E/S181E (SSEE) IKKβ were created. One main problem when making stable cells, by the classic method of random integration of the gene into the cells genome, is that the integration can occur anywhere in the genome and at any number of locations. This is particularly problematic when comparing between different stable cell lines. For example, if the mutant form were integrated at more locations or at locations with higher transcriptional activity than the wild-type then the two cells would have a different level of overexpression which could skew results. In order to avoid this, the Gateway Flp-In system was used. The work-flow for this is detailed in Fig.4.12. Briefly this involves the integration of a single FRT site within a cell line (in this case A375 cells), as well as cloning the gene of interest into a gateway vector with an FRT site. When the vector is transfected into the cells along with the pOG44 enzyme vector, the enzyme catalyses recombination of between the FRT sites. This means that every time a gene is transfected into the parental cell recombination will happen only once at the same site in the genome. In this experiment A375 cells with a single stably integrated FRT site were used and were made in the lab by Khaldoon Al-Samman.

As can be seen in Fig 4.13A integration of the wt-IKKβ and KM-IKKβ into the A375-FRT cells lead to a similar level of overexpression. However, slightly reduced overexpression of the SSEE-IKKβ form is observed in comparison to wt and KM IKKβ. As the Flp-In system means this should not be due to differential gene integration it suggests that this could be due to differential regulation of active and inactive forms of the kinase. IKK degradation is only recently being understood and may involve both proteasomal degradation and autophagy (*248, 249*). Whilst it would be imagined that activation would, if anything, lead to increased stability of the protein, such pathways often have negative feedback mechanisms which act to shut off the pathway after a period of time. For example IKK activation of Nf-kB via the classical pathway is transient and when IKKβ is activated by phosphorylation this leads to auto-phosphorylation near the C-terminus which inhibits activity (*247*). This result potentially suggests that increased kinase activity could also lead to negative regulation of protein stability.

As can be seen in Fig 4.13 B overexpression of wild-type, KM and SSEE IKKβ lead to a slight increase in the amounts of MDM2 compared to the parental cell line. With IMD0354 treatment no clear difference in MDM2 levels with overexpression of the different IKKβ mutants is perceptible. The levels of MDM2 however should really be compared between cell lines overexpressing the different mutants as these cells have undergone the same selection procedure. It would be anticipated therefore that if overexpression of IKKβ was leading to an increase in MDM2 that overexpression of SSEE IKKβ would led to substantially higher levels of MDM2 than KM IKKβ. Overexpression of SSEE IKKβ appeared to lead to very slightly higher levels of MDM2 than wt IKKβ or KM IKKβ. However, this was not substantial and thus it cannot be concluded at this time that overexpression of IKKβ leads to an increase in MDM2 protein levels. It should be noted that just because IKKβ inhibition reduces MDM2 it does necessarily mean that an increase in IKKβ would led to an increase in MDM2 and regulatory mechanisms may exist to prevent an increase in MDM2.

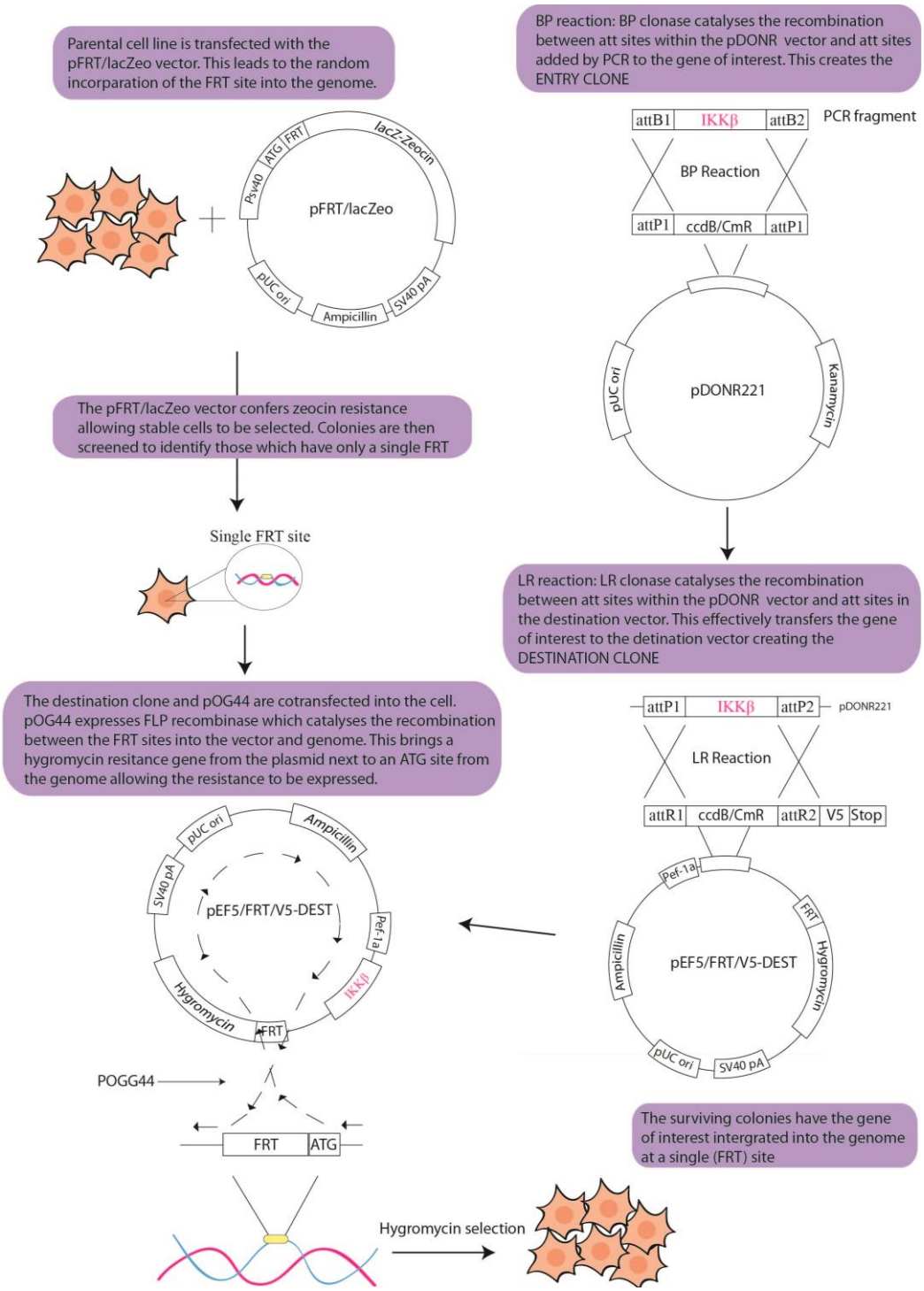

Fig 4.12 Work-flow of the creation of stable cell lines. FRT-lac Zeo is transfected into the chosen cell line. Colonies which have one integrated FRT site are then selected, this is termed the parental cell line. In parallel to this the destination clone is created; the gene of interest (GOI) is cloned into an entry vector such as pDONR221 by BP reaction. The GOI is then transferred to the destination vector (pEF5/FRT/V5-DEST) by an LR reaction. The destination clone and pOGG44 is then transfected into

the parental cell line. pOGG44 then catalyses the recombination between the FRT sites allowing the stable integration of the GOI into the genome at a single site.

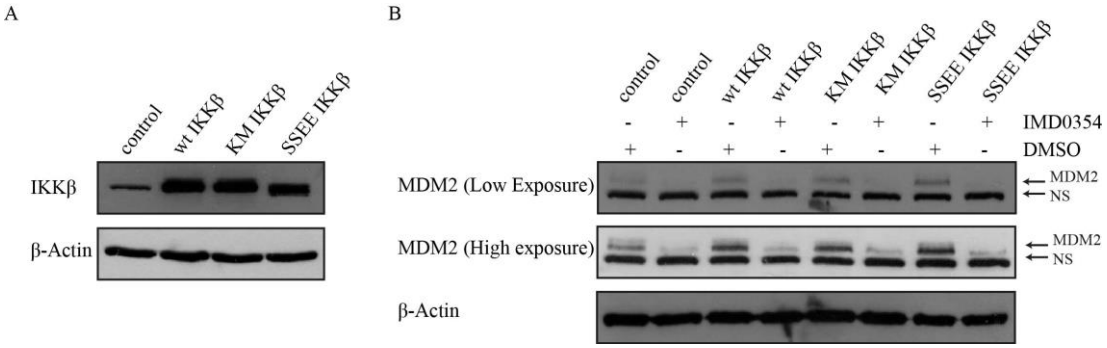

Fig 4.13 IKKβ overexpression does not lead to substantial changes in the levels of MDM2. (A) A375 cells overexpressing wt-IKKβ, K44M-IKKβ (KM) and S177E/S181E-IKKβ (SSEE) were made using the Gateway Flp-In system. Cell lysates (20µg/well) were then assessed by western blotting to determine relative IKKβ levels using an anti-IKKβ antibodies. Equal loading was also assessed using anti-β-actin antibody. **(B)** Cells overexpressing IKKβ (wt, KM, SSEE) were treated with 5µM IMD0354 or DMSO for 2Hrs. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody 2A10. Equal loading was also assessed using anti-β-actin antibody.

4.2.9 Increased cell density reduces MDM2 and D type cyclins.

Research from Moshe Oren's group has shown that the p53 pathway can be attenuated by high density (*250*) in addition to this previous work within the lab has shown that MDM2 levels specifically can be effected regulated by cell density (*unpublished data*). As shown in Fig 4.14, MDM2 is likewise regulated by cell density in T47D cells with higher densities; leading to a reduction in MDM2 levels. It was investigated whether other proteins altered by IKK inhibition are also affected by this mechanism. As can be seen in Fig 4.14 cyclin D1 is initially increased as cell density increases. This is expected as cyclin D1 is required for the cell cycle and so will be at higher levels in proliferating cells. Cyclin D1 is thought to be required for, and is increased during G1 and G2 phase but is reduced during S phase of the cell cycle (*251*). As the cells were asynchronous it can be expected that cyclin D1 expression would be higher in the cells overall when they are replicating. As cells reach a higher level of density the cells will have stop dividing due to contact inhibition. Interestingly cyclin D1 is lost at the same time-point at which MDM2 is reduced.

A number of kinase inhibitors have been shown to be able to overcome the reduction in MDM2 as a result of density (Y Lin. *Unpublished data*). It could be speculated that manipulation of these same pathways could also overcome MDM2 reduction as a result of IMD0354 treatment, therefore these kinase inhibitors (detailed in Table 4.1) were tested by in cell western for their ability rescue MDM2 loss after IMD0354 treatment. As can be seen from Fig 4.15A only one of these inhibitors, Ki-8751, which inhibits VEGFR, appeared able to increase MDM2 levels. Further analysis by in cell western (Fig 4.15B) revealed that Ki-8751 alone does not lead to significant changes in MDM2 levels but treatment of T47D cells with 50µM or 25µM Ki-8751 appears to be able to partially overcome the loss of MDM2 resulting from IKKβ inhibition (PVal= 0.002, 0.024 respectively). Western blotting was then used in order to try to validate this result; however, this did not demonstrate any rescue of MDM2 levels resulting from Ki-8751 treatment.

Potentially, this could result from the better sensitivity of in cell western in comparison to western blotting (*205*); however this remains undetermined.

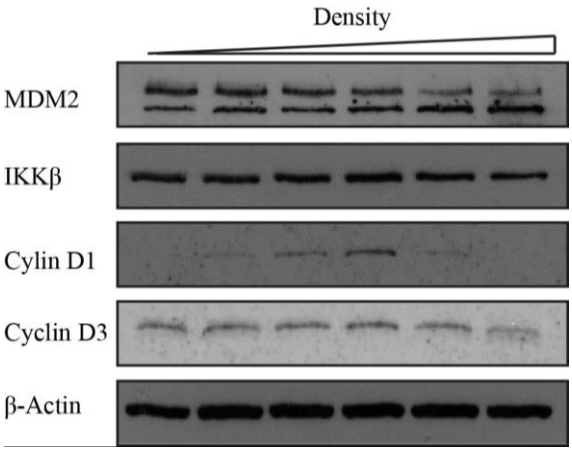

Fig 4.14. High cell density leads to a reduction in MDM2 and D-type cyclins. T47D cells were grown at 6 different densities. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2, IKKβ, cyclin D1 and cyclin D3 levels using anti-MDM2 (2A10), anti-IKKβ, anti-Cyclin D1 and anti Cyclin D2 antibodies. Equal loading was also assessed using anti-β-actin antibody.

Table 4.1. Compounds identified as potentially reducing MDM2 loss at high density

Name	**Target kinase**
ZM 306416 hydrochloride	VEGFR
Compound 401	DNA-PK
PD 198306	MEK
GW 583340 dihydrochloride	EGFR
CGP 53353	PKC
Ki 8751	VEGFR
Aminopurvalanol A	cdk
SD 208	TGFbR1
EO 1428	p38 MAPK

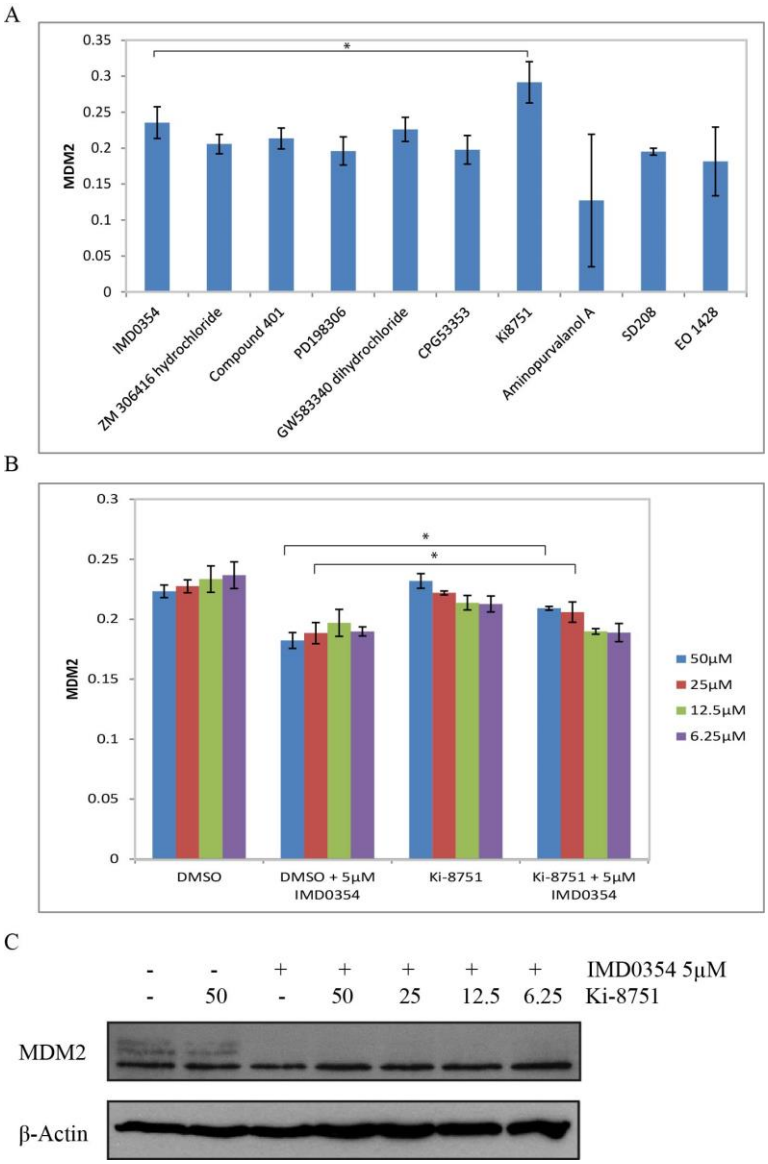

Fig 4.15 In cell western but not western blotting suggests Ki-8751 can partially rescue the loss of MDM2 by IMD0354. (A) T47D cells were treated with 5µM IMD0354 as well as 50µM of the indicated compound for 2Hrs. MDM2 levels were then quantified by in cell western using the MDM2 specific antibody 4B2. Columns show means of results in triplicate and error bars indicate standard deviation. * denotes a significant change, assessed by T-test, in MDM2 level compared to IMD0354 treated cells (PVal>0.05) **(B)** T47D cells were treated with IMD0354 and Ki-8751 as indicated for 2Hrs. MDM2 levels were then quantified by in cell western using the MDM2 specific antibody 4B2. Columns show means of results in triplicate and error bars indicate standard deviation. Statistical significance between DMSO versus Ki-8751 and IMD0354 Versus IMD0354 + Ki-8751 was assessed by T-Test. * denotes a significant change in MDM2 level (PVal>0.05) **(C)** T47D cells were treated with IMD0354 and Ki-8751 as indicated for 2Hrs. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody.

4.2.10 IMD0354 does not increase MDM2 turnover rate

IKKβ inhibition could potentially lead to a reduction in the levels MDM2 by a number of mechanisms e.g. it could lead to an increase in protein degradation or lead to a reduction in protein synthesis. MDM2 is normally degraded via the ubiquitin-proteasome system and therefore it was investigated whether inhibiting the proteasome would prevent the loss of MDM2. Alteration of MDM2 stability is thought to be an important mechanism in the regulation of the p53 pathway. For example after DNA damage MDM2 is phosphorylated by CK1 which promotes it's ubiquitination by β-TRCP, leading to rapid degradation (*252*). This allows p53 activation by reducing its degradation by MDM2. As shown in Fig 16A the proteasome inhibitor MG132 was only capable of a partial rescue of MDM2 levels if cells were treated with MG132 and IMD0354 at the same time. If pre-treated with IMD0354 then rescue was not possible. This implies that once the MDM2 that is present in the cell is degraded, the levels of MDM2 cannot be re-established by blocking degradation. This means that degradation does contribute to the reduction of MDM2, but such reduction does not result from changes to proteasomal degradation.

However there are multiple mechanisms of degradation within a cell and it is therefore possible that MDM2 is lost as a result of trafficking into a different degradative system. In order establish whether the rate of degradation is affected by entering a different degradation system the turnover rate of MDM2, with and without IMD0354, was assessed. As can be seen in Fig 16B-C the rate of turnover was not increased as a result of IMD0354 but turnover rate appeared to be marginally decreased. This shows that changes in degradation rate is not a major mechanism by which IMD0354 reduces MDM2 levels.

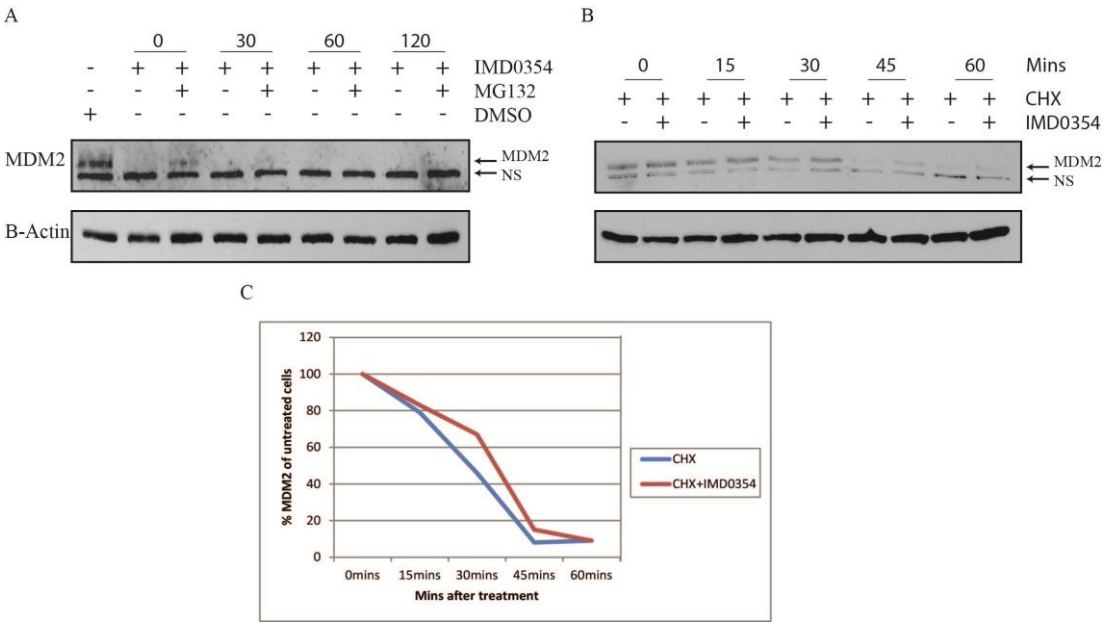

Fig. 4.16. IMD0354 does not increase the turnover rate of MDM2. (A) T47D cells were treated with MG132 and IMD0354 for 2Hrs. Cells were additionally pre-treated with IMD0354 for an additional 0, 30, 60 or 120mins. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody. **(B)** T47D cells were treated with cycloheximide (CHX) with and without IMD0354 for the indicated time. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody. **(C)**. The graph shows the quantification of MDM2 levels in **(B)** normalised to B-actin levels expressed as a % of untreated cells (0mins time-point). The western blot image was quantified using ImageJ software.

4.2.11 MDM2 mRNA levels are reduced by IMD0354 treatment

As the degradation of MDM2 is not increased by IMD0354 this implies that *de novo* production must be being inhibited to bring about MDM2 loss. This is consistent with the fact that MDM2 loss resulting from IMD0354 treatment occurs at a rate roughly equal to its rate of turnover. Proteins are made in a number of distinct steps; first mRNA is transcribed from DNA, the mRNA is then processed and relocated to the transcription machinery and finally the protein is produced by transcription.

The levels of MDM2 are known to be regulated at the level of transcription. MDM2 transcription is most typically thought to be controlled by p53 (*163*), however this study has demonstrated that the reduction in MDM2 levels resulting from IMD0354 is independent of p53. Nonetheless, this does not discount that the idea that transcriptional changes could be important, as MDM2 transcription is not solely a result of p53 activity, and other transcription factors are known to promote MDM2 transcription (*27*). Therefore, the response of MDM2 mRNA levels to IMD0354 treatment was investigated. MDM2 mRNA levels were assessed by qPCR and were compared with β-Actin mRNA levels as a control. The percentage change in mRNA levels was analysed by the $2^{-\Delta\Delta Ct}$ method (Detailed in the Methods Chapter). As can be seen in Fig 4.17A, mRNA was significantly reduced by IMD0354 and ~40% reduction was observed. The reduction in mRNA levels appears to be less acute in comparison to the reduction in protein level (Fig 4.16B) however the dynamic range of these two techniques likely differs. Furthermore different transcripts of MDM2 lead to different rates of synthesis meaning that a relatively small change in mRNA levels could lead to a bigger change in protein level (*25*). Nevertheless the relatively small change in mRNA levels does suggest that other contributing mechanisms to the reduction of MDM2 protein level are possible and cannot be ruled out at this time.

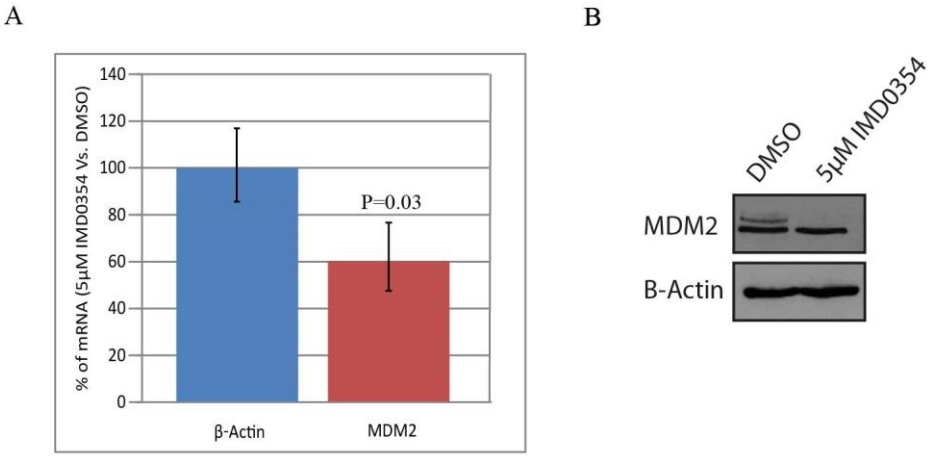

Fig. 4.17. IMD0354 leads to a reduction in MDM2 mRNA concomitant with a reduction in MDM2 protein. (A) T47D cells were treated with 5µM IMD0354 for 2Hrs. MDM2 mRNA levels were then assessed by qtPCR. Relative changes in mRNA levels were analysed using $2^{-\Delta\Delta Ct}$ method. The statistical significance of the change in ΔΔCt between β-actin and MDM2 was assessed by T-Test and the P-Values indicated. **(B)** T47D cells were treated with 5µM IMD0354 for 2Hrs. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody.

2.3 Discussion

This study shows that the IKKβ inhibitor IMD0354 leads to a reduction in a number of proteins including mtp53, MDM2, p21 and Cyclin D. It also further defines IKKβ as an important factor for the maintenance of the cellular levels of MDM2. IKK is a multi-subunit complex with known roles in allergy, inflammation, immunity and cancer (*211, 212*). IKK is best known as an activator of Nf-κB (See Fig 4.1); a family of related transcription factors (*99, 100*). However, in addition to activation of Nf-κB, IKK can also phosphorylate a variety of other substrates (*211*). The IKK complex is composed of IKKα (IKK1), IKKβ (IKK2) and nemo (IKKδ) subunits. IKK complex can be made up of various combinations of these three subunits. Not all complexes necessarily contain all three and these different complexes are known to contribute to different pathways. For example, activation of Nf-κB (Fig 4.1) by the classical pathway relies on complexes composed of 2 nemo molecules and a hetero or homodimer of IKKα and/or IKKβ, whilst activation by the non-classical pathway relies on only IKKα (*212*). α and β subunits both have kinase activity whereas nemo has only a regulatory role (*212*).

Whilst this study initially set out to characterise IKKβ in terms of its ability to regulate p53 levels it quickly became apparent that IKKβ inhibition by IMD0354 can lead to the reduction of a number of other proteins. This work has largely used MDM2 as a surrogate marker of IKKβ regulated proteins as it responds rapidly after IKKβ inhibition. The order in which the different proteins (p53, MDM2, p21) are lost appears to reflect the relative stabilities of each protein suggesting that the more rapidly turned over proteins will be lost first.

Identification of factors affecting MDM2 such as IKKβ is critical as MDM2 is considered to be an oncogene and so its loss is considered to be beneficial for therapy (*108, 233, 253*). When MDM2 levels are reduced in cell lines using siRNA this can lead to reduced cell growth and apoptosis independent of p53 status (*234, 235*). MDM2 is considered to be oncogenic via a number of mechanisms and it is frequently seen to be overexpressed in cancer cells (*26*). Originally its oncogenicity was thought to be due to its ability to supress p53 and prevent its action as a

tumour suppressor, however more recently it has become apparent that it has a number of other mechanisms are also responsible for its action (*235*). MDM2 is now considered to have many p53 independent roles in a number of cellular processes including cell cycle control, proliferation and differentiation (*232*).

This study mainly focussed on using IMD0354 to inhibit IKKβ. This IKKβ specific inhibitor was first reported in 2004 and since been used in various studies to investigate the importance of IKKβ in cancer pathways (*229, 230*). Experiments in mice have shown that IMD0354 does not lead to general toxicity and IMD0354 has been proposed to be an important line of inquiry for future study as it may potentially be useful in treating cancer as well as other disorders (*229, 230, 240*).

Although mainly focussed on IMD0354, in total three small molecule inhibitors targeting IKKβ; IMD0354, IKK16 and BMS-345541 were shown in this study to reduce levels of MDM2. IKKα and IKKβ can function as both hetero and homodimers, but the results presented here suggest that inhibition of IKKβ is sufficient to lead to the reduction in MDM2. A number of studies have shown that IKKβ appears to have greater activity than IKKα in the classical pathway which is reliant on both isoforms (*241, 242*). However, the relative contribution of each is hard to determine and it has been suggested that inhibition of one may well affect activity of the other (*241*). IKKβ inhibitors tend to also show activity towards IKKα albeit at a higher concentration (*244, 245*). This study mainly focused on using chemical inhibitors as therapy currently largely relies on such therapeutics. Unfortunately, no specific IKKα inhibitors are to our knowledge commercially available and so the effect of inhibiting IKKα was not specifically assessed. Therefore, it cannot be ruled out that IKKα may also be able to contribute to MDM2 stability.

siRNA knock-down of IKKβ supports the results from chemical inhibitor data as siRNA knock-down of IKKβ similarly lead to a reduction in MDM2. However, there were notable differences in the extent of MDM2 reduction in the two techniques. IMD0354 caused a substantial loss of MDM2 whereas IKKβ targeting siRNA lead to a more subtle reduction. There are a number of potential reasons for this. Firstly,

siRNA leads to target knock-down and is unable to lead to a total loss of IKKβ protein. Basel levels of IKK activity may not require 100% of the protein present. The IKK pathway is thought to be activated by phosphorylation of IKKβ (*247*) and the level of IKK present in the cell may not be the limiting factor with base level activity. Therefore, reduction observed with siRNA may not bring about as dramatic a change as with a chemical inhibitor, which is likely to be in excess. The alternative is that the fact that inhibition of IKKβ by siRNA is a slower process, reducing IKKβ over days rather than minutes, may mean that the cell has time to adapt to the changes occurring. MDM2 levels are tightly regulated and siRNA experiments have shown in many cases that excessive reduction of MDM2 in cancer cells is not tolerable (*235, 254*) and thus, it is possible that the cell may have an adaptive responsive to an indirect reduction in MDM2.

The levels of MDM2 can be regulated by a number of processes including the density of cells (*data not published*) and, as also shown here, MDM2 is reduced at higher densities. Analysis of a number of compounds, that have previously been shown to reduce MDM2 loss at high density (*data not published*), by in cell western technique showed that inhibition of VEGFR by Ki-8751 may partially rescue the reduction in MDM2 by IMD0354 treatment. As growth factors including VEGF are secreted it is logical that at higher densities more VEGF may be present in the media. However it is less clear how VEGF could contribute to the IMD0354 mediated down-regulation of MDM2. IKK has been shown to be an upstream regulator of VEGF (*255, 256*) meaning that IKK inhibition should also act to inhibit VEGF expression. MDM2 has also been shown to positively regulate VEGF via HIF-1 (*257*), and there does not appear to be any established mechanisms of VEGF regulation of MDM2. Western blotting was unable to reproduce the rescue of MDM2 by Ki8751. This could be due either to intrinsic differences in the available/extractable pools of MDM2 in the two techniques or, could potentially reflect the greater sensitivity of the in cell western technique (*205*). The results from the in cell western technique showed that MDM2 recovery was modest and protein levels were still substantially below the levels seen in untreated cells. Such a small recovery therefore may be

undetectable by western blotting. This does however suggest that if VEGFR does play a role in the dissemination of this pathway it is only a very minor one and is non-essential. It should also be noted that a significant recovery was only seen with a relatively high concentration of Ki8751.

The study showed that IKKβ inhibition by IMD0354 leads to a reduction in MDM2 protein which is concomitant with a reduction in MDM2 mRNA levels. The turnover rate of MDM2 protein was not increased by IMD0354 but in fact appeared to slightly decrease the rate of degradation. Potentially the mechanism by which IKKβ prevents MDM2 production could induce an adaptive response whereby the loss of existing protein is slowed. Quantitative PCR suggest that the reduced level of MDM2 may in part be due to a reduction in mRNA levels. Together these results suggest that IKKβ is required for maintaining mRNA levels, either by sustaining transcription or by preserving the stability of mRNA. However, mRNA levels were only reduced modestly (by ~40%), whilst protein levels appear to be much more dramatically reduced. Differences in the dynamic ranges of the two techniques could be contributing the observed disparity; however other mechanisms of regulation may be of importance and were not excluded in this study. Another potentially important mechanism could be mRNA localisation and storage. Future work could assess mRNA localisation using fluorescent *in situ* hybridisation (FISH). It may also be useful in future work to define the rates of transcription using a nuclear run-on assay.

The relationship between IKK and the p53 pathway including MDM2 is complicated. Whilst it has been shown there are a large number of ways in which the pathway can interact (*39*) the dominant outcome is difficult to predict. The importance of IKK in MDM2 regulation has previously mainly been investigated in terms of p53 i.e. investigating the effect of IKK perturbation on MDM2 as a consequence of, or subsequent to, p53 change. As such, this axis has been little investigated in mtp53 cells. MDM2 has been previously been shown to be reduced as a consequence of IMD0354 treatment. Tanaka et al. (2006) showed that IMD0354 lead to cell cycle arrest and apoptosis by reducing the levels of cell cycle regulators

and anti-apoptotic proteins (*229*). One of the cell cycle regulators they considered was MDM2 and showed it was reduced in MDA-MB-231 cells at concentrations down to 5μM, but no reduction was seen at 1μM. This study found that in T47D, H1299, A375 and MDA-MB-231 cells MDM2 was reduced as low as 400-800nM. The comparison of western blots shows that the reduction in MDM2 in MDA-MB-231 cells as a result of IMD0354 treatment may be more subtle; however comparison between western blots is problematic and within this study any difference between sensitivities cannot be confirmed. They concluded that the changes in MDM2 were not due to any alteration in p53 as the p53 in this cell line is mutant and no changes in p53 level were observed. Whilst this study similarly concluded that MDM2 reduction was p53 independent a reduction in p53 levels was observed. Such differences could potentially reflect differences in the cell lines used (MDA-MB-231 versus T47D and A375 cells). Although alternatively it is possible that the turnover rate of mtp53 in MDA-MB-231 is too slow to observe any decrease within the time period used and that the effects of IMD0354, but not the kinetics, were the same as in the cell lines used in this study.

The literature shows that manipulation of IKK or Nf-κB could lead to changes in MDM2 via a number of different mechanisms. Lau et al. (2012) showed that activation of the IKK pathway via disruption of cIAP2 could lead to post-translational modifications of MDM2 enhancing its activity (*222*). Post-translation modifications leading to altered stability, do not however, appear to be important within this study as turnover of MDM2 was not increased upon IKK inhibition. However this study investigated chemical inhibition of basal activity rather than changes after induced Nf-kB activity so such differences are not at odds.

Consistent with the work presented here other studies have also shown that inhibition of the IKK pathway can lead to changes in the levels of transcription. Tergaonkar et al. (2002) observed that in IKKβ-/- MEFs MDM2 levels were lower than in those reconstituted with IKKβ (*220*). They also showed that IKKβ-/-MEFs had reduced MDM2 mRNA levels; again this is consistent with the findings in this study. However one major difference was that they found that increasing MDM2 levels by

IKKβ reconstitution was dependent on p53. However Tergaonkar et als study focused on using MEFs and it is highly likely that different regulatory mechanisms exist in MEF compared to cancer cell lines. Furthermore they largely relied on knock-out technology whereas this study mainly, although not exclusively, investigated chemical inhibition. They also found that a lack of IKKβ in MEFs promoted the stabilisation of p53 in response to doxorubicin. Interestingly whilst it was shown in this study that IMD0354 can reduce wtp53 the effect was not consistent across different concentrations. Within the previous chapter screening of the Tocris kinase inhibitor library did not identify IMD0354 as a p53 activator and furthermore such a dramatic reduction in MDM2 seems inconsistent with p53 activation. However, within this chapter the effect of IMD0354 on wtp53 activation was not specifically assessed and further work would be required in order to conclusively show changes in p53 activity with IMD0354.

A study by Busuttil et al. (2010) also found that in T-cells Nf-κB can lead to increased transcription of MDM2 (*221*). Consistent with the data here they did not find that this process was dependent on p53 but rather suggested that transcription may result from direct binding of Nf-κB to the P1 promoter.

In conclusion, IKKβ is known to be responsible for negatively regulating the stability of IKB and this research supports the idea that IKKβ can also lead to positive regulation of a separate subset of proteins including mtp53, D-type cyclins and MDM2 (Fig 4.18), all of which have been shown to contribute to oncogenesis. This potentially occurs by a mechanism which increases the levels of mRNA, although the mediators of this effect remain unidentified. As discussed an exploration of the literature does provide some potential possibilities, however such an approach can bias our investigation towards better understood mechanisms and leave other contributing pathways unidentified. As such, the following chapter will use a proteomics approach to explore mediators and co-regulated proteins within this pathway.

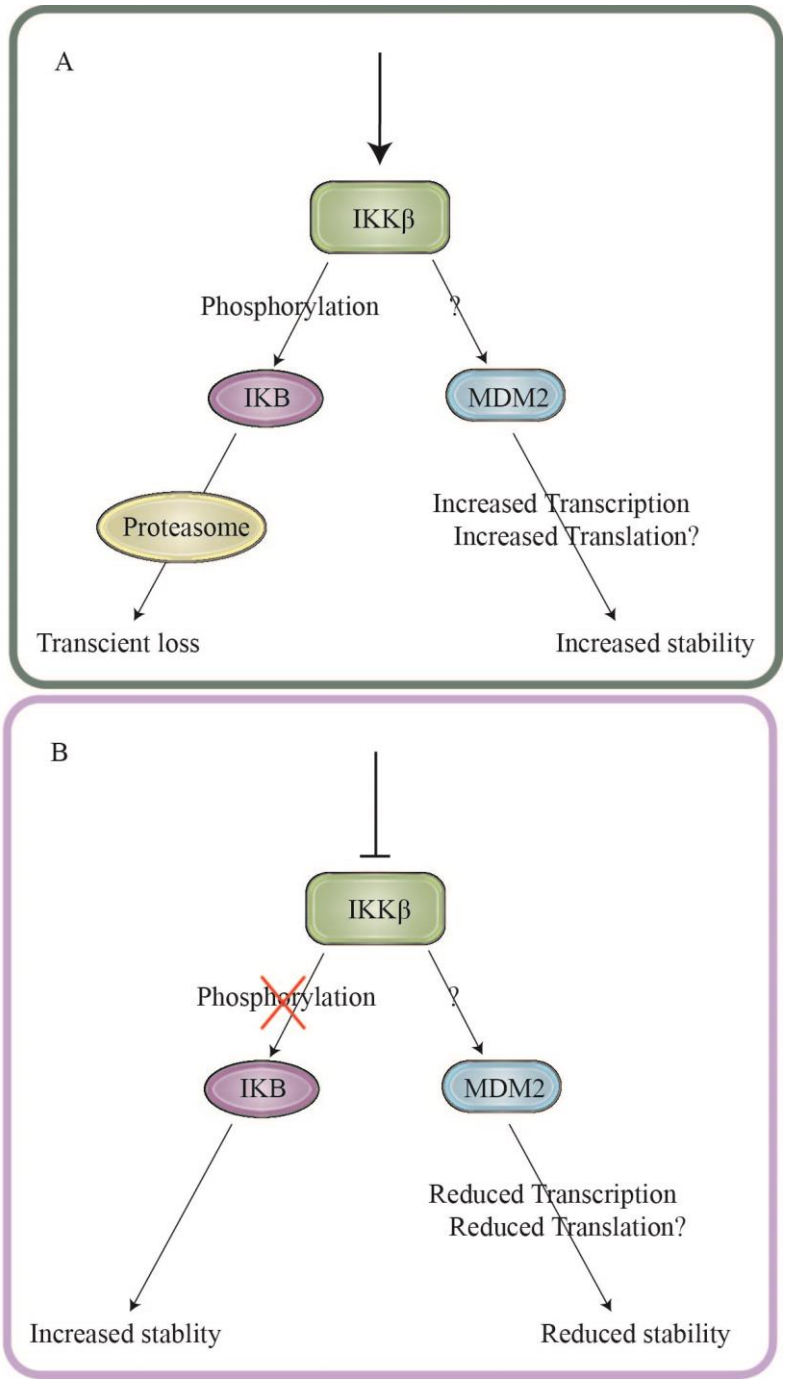

Fig 4.18 Model of the regulation of IKB and MDM2 by IKKβ (A) IKKβ phosphorylates IKB leading to its degradation by the proteasome. Conversely IKKβ promotes the stability of MDM2 potentially via increased transcription and translation. **(B)** IKKβ inhibition leads to reduced IKB phosphorylation allowing IKB to escape to its degradation by the proteasome. Conversely IKKβ inhibition reduces the stability of MDM2 potentially via decreased transcription and translation.

Chapter 5

Proteomic investigation into the effect of the IKKβ inhibitor, IMD0354 on T47D cells

5. Proteomic investigation into the effect of the IKKβ inhibitor, IMD0354 on T47D cells.

5.1 Introduction

The previous chapter investigated the effect of IKKβ inhibition on the p53 pathway. This mainly focused on characterising the effect of the IKKβ inhibitor IMD0354 and showed this inhibitor lead to a reduction in MDM2 protein levels consistent with the effects of other IKKβ inhibitors as well as RNAi inhibition. In addition it was shown that inhibition of IKKβ using IMD0354 led to the reduction of mtp53, p21 s as well as D-type cyclins (D1 & D3). This chapter aims to further explore the IKKβ pathway in terms of the effects on the proteasome after IKKβ inhibition. Specifically, this chapter aimed to identify other proteins regulated by IMD0354 treatment to identify both co-regulated factors with, and mediating factors of, MDM2 reduction. Furthermore this will help address how specific the requirement of IKKβ is in reducing p53 regulated proteins and Cyclin Ds; i.e. does it maintain a limited number of pathways or does it have a more general effect on the proteome. IKKβ is known to affect a wide number of proteins, directly by phosphorylating substrates and indirectly as many of these substrates are transcription factors (*211*). In an attempt to take an unbiased approach to investigating this pathway, proteomic analysis was employed.

Due to the fact that there is so much cross talk between different pathways within the cell it is becoming increasingly apparent that the response of the proteome as a whole is important in analysing outcomes of pathway perturbation. Such analysis is termed proteomics; a phase which was coined by Marc Wilkins at Macquarie University (1994) (*258*). Proteomic analysis is used for the discovery of new biomarkers for cancer and other disease, exploring the interactome of a given protein or for identification of pathways regulated by a given manipulation (*259-262*).

Analysing the proteome however is not simple, and whilst significant progress is being made in proteomic methods no technology exists which can detect

and quantitate all proteins within the cell. Current proteomic analysis typically relies on mass spectrometry (MS) techniques. For the analysis of complex samples proteomic techniques rely on the extraction of proteins and digestion of these proteins to produce peptides which can be analysed by Tandem MS (MS/MS). Mass-spectrometry identifies the mass/charge ratio of the molecule which can be used to identify the molecule or compound in question. Due to the complexity of peptides, tandem mass spectrometry is required for identification. Once samples have been ionised they are analysed by the 1st MS in order to identify the precursor or parental peaks. This gives the mass-charge ratio of the whole peptide, due to the existence of many possible peptides with variable lengths this information is not enough for identification of the peptide/molecule. Therefore peaks are selected and the peptides are subjected to fragmentation to identify the secondary or daughter peaks. This second MS will provide identification of the peptide in terms of its amino acids. This is possible as there are only a limited number of amino acids with different masses. The differences in mass between each of the y and b ions will be the mass of the specific amino acid (Fig 5.1). For example, if a mass shift of 71 is observed this would be most likely explained by the loss of an alanine residue.

Fractionation of the sample is also critical to ensure a maximal identification of proteins within the sample (*263, 264*). To some degree, fractionation will also aid in the identification of lower abundance species as each of the fractions will be less complex (*263, 264*). Fractionation can be achieved by a number of techniques, including subcellular fractionation, gas phase fractionation or, as used here, strong cation exchange (SCX).

Proteomic analysis generally requires comparison of two or more samples *i.e.* comparison of a control or base level sample versus a diseased or manipulated sample. In order to do this a number of labelling techniques have been developed which allows peptides from different samples to be labelled and then analysed together by MS. Two different labelling techniques were used in the experiments presented here. The first experiment used a common post-digestion labelling system; isobaric tags for relative and absolute quantitation (iTRAQ) (*265*). iTRAQ

tags are covalently attached to digested peptides of the different samples. There are 8 different iTRAQ tags typically available, however here only four tags, or a 4-plex experiment was conducted. One advantage of fewer samples analysed is that the mixture is less complex and so generally a greater depth of data is achieved. The individual iTRAQ tags have the same overall mass but are composed of different weight reporter mass groups and variable balancer mass groups which act to keep total mass the same (*265*) (Fig 5.2A). As the total mass of the iTRAQ tags is the same, the same peptides from different treatment groups, and so different labels, will be detected together in MS1. When fractionated, the reporter group will is released and the relative intensities of each tag will indicate the relative amount of each peptide within the sample. Fig 5.2B shows an example of this within one of the iTRAQ data sets presented here. The graph shows the Mass/change peaks obtained from fractionating the peptide DELADEIANSSGK. As can be seen at the lower mass/charge end there are 4 peaks corresponding to mass of the labels used (114-117). The intensities of these indicates the relative abundance of this peptide in each sample.

Another commonly used labelling method which is utilised in this study is stable isotope labelling by amino acids in cell culture (SILAC) (*266*). SILAC involves growing cells in media containing either light or heavy isotopes for example commonly used isotopes are $C^{12/13}$ and $N^{14/15}$. As the cells grow they will use the labelled elements (e.g. $C^{12/13}$ and $N^{14/15}$) from the media for protein synthesis. This means that after a period of time all proteins within the cell will be either "light" or "heavy" labelled depending on the media used. This results in mass shift in otherwise identical peptides that can be detected by MS1. Commonly used light and heavy isotopes are $C^{12/13}$ and $N^{14/15}$. SILAC is a particularly useful labelling method for studying phosphoproteomics as it means that after labelling all samples can be processed together (*267*). This means that any sample handling that could affect the sample should affect both equally; this is particularly useful when using an enrichment technique such as in phosphoproteomics as significant sample processing is required. However, SILAC does have the disadvantage that analysis is

limited to 3 samples; “Heavy”, “Medium” and “Light” labelled samples. Furthermore, SILAC cannot be used in clinical samples due to the impracticality of labelling patients.

As proteomics has advanced it is becoming an increasingly useful technique to analyse proteins with specific modifications. For example, there is significant use of MS in phosphoproteomics and glycoproteomics (*267, 268*). In terms of phosphoproteomics, MS can be used to infer activity within a specific pathway or the activity of a given kinase (*262, 267, 269*). A number of methods have been developed to extract phosphorylated peptides or proteins from total peptides and commonly used techniques include Titanium dioxide (TiO_2) enrichment and immobilized metal affinity chromatography (IMAC) (*270, 271*). In the IMAC technique, phosphopeptides are extracted by the interaction of the negatively charged phosphate groups with positively charged metal ions (Fe^{3+}, Ga^{3+}, and Al^{3+})(*270, 272*). Phosphopeptides can also interact with metal oxides, a fact exploited by TiO_2 bead enrichment (*271*). TiO_2 is generally thought to be a more robust technique which shows greater affinity to mono-phosphorylated peptides over multi-phosphorylated peptides, on the other hand IMAC is better at enriching for multi-phosphorylated peptides (*273*) (Information for supplier: http://www.piercenet.com/browse.cfm?fldID=87231F16-AA7F-A46A-2046-D3B47B35012D).

In this study, mass spectrometry will be used to discover the changes to the proteome resulting from drug treatment with IMD0354. Two approaches are explored to investigate the effects of IMD0354. The first sought to identify proteins which are up or downregulated in response to IMD0354 treatment utilising isobaric tags for relative and absolute quantitation (iTRAQ). The second was a study to identify changes to the phosphoproteasome resulting from IMD0354 treatment which could provide leads as potential IKKβ substrates. This was conducted by phosphoenrichment by TiO_2 beads of peptides from the tryptic digest of protein from SILAC labelled cells.

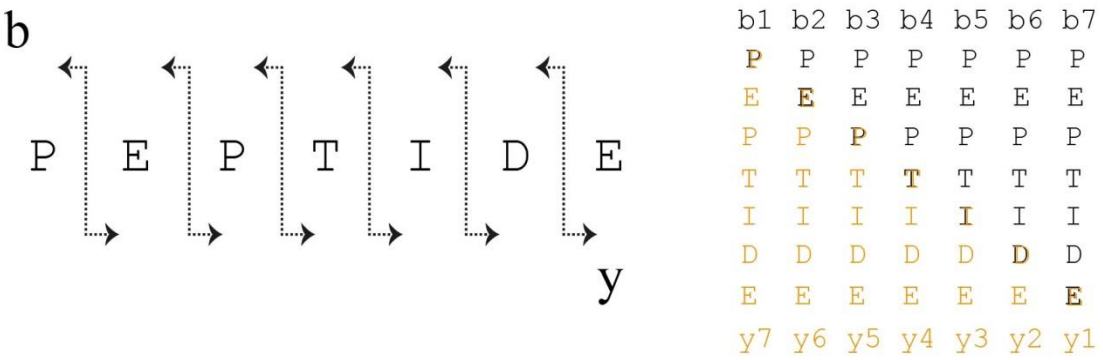

Fig 5.1 Peptide sequencing by Tandem MS. During Tandem mass spectrometry primary peaks are identified during MS1 and represent whole ionised peptides. These peptides are then fractionated into y and b ions as shown. The mass difference between the ions enables identification of amino acids by their weight. Figure adapted from Ross et al. (2004).

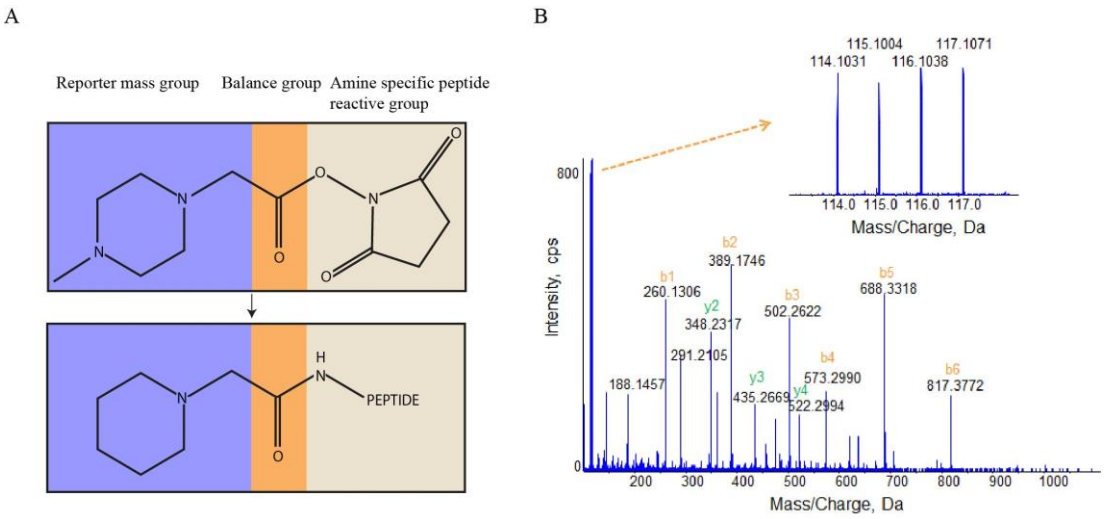

Fig 5.2. iTRAQ MS/MS. (A) iTRAQ tags are used to label different samples within mass spectrometry experiments. These tags contain 3 groups, reporter, balance and peptide reactive groups as shown in the top panel. In the 4plex set of tags the reporter group has a mass of between 114-117Da, the total tag mass is kept the same with a balance group of between 28-31Da. The Reactive group allows the tag to be covalently bonded to the peptides as shown in the bottom panel. In MS1 the same peptide with different tags will form one parental peak. Figure adapted from Ross et al. (2004). **(B)** During tandem mass spectrometry the peptides are fractionated at the MS2 stage, at this point the reporter group on the tags will break off and the relative intensities of each tag will allow relative quantitation of the peptides in each group. The expanded section shows the tag reporter group intensities. The peptide shown is DELADEIANSSGK from Myosin-9 (546.28 m/z).

5.2 Results

5.2.1 Preliminary analysis of samples

Primarily, this study was designed to identify proteins which were regulated by IKKβ inhibition by proteome analysis. In this pilot screen, samples were prepared for analysis by iTRAQ by growing T47D cells to ~80% confluency and treating with 5μM IMD0354. Cells were then harvested at 0, 30, 60 and 120 minutes after treatment and prepared for iTRAQ analysis. Previous work in this study has shown that this treatment window should lead to the reduction of the MDM2 protein level (Fig 4.4C). Whilst this treatment was never seen to fail to reduce the levels of MDM2 it was considered prudent to check this in samples used for mass spectrometry analysis. As shown in figure 5.3A, MDM2 protein expression is, as expected, reduced overtime in the samples used.

A second aim of this study was to identify changes to the phosphoproteome resulting from IMD0354 treatment. This may provide potential IKKβ substrates relevant for this system; substrates phosphorylated by IKKβ would be expected to demonstrate reduced levels of phosphorylation upon IKKβ inhibition. For this experiment, cells treated for 120mins with IMD0354 were compared to cells treated with drug vehicle, DMSO. For this approach, cells were labelled prior to treatment using SILAC. After labelling, cells were then grown to ~80% confluency and treated with 5μM IMD0354 or DMSO for 120mins. Again, prior to MS/MS analysis, samples were checked to ensure that IMD0354, had, as anticipated led to a reduction in MDM2 protein expression. As can be seen in Fig 5.3B MDM2 had been reduced by IKKβ inhibition in this sample. This also indicates that SILAC labelling did not affect IMD0354 mediated reduction of target proteins.

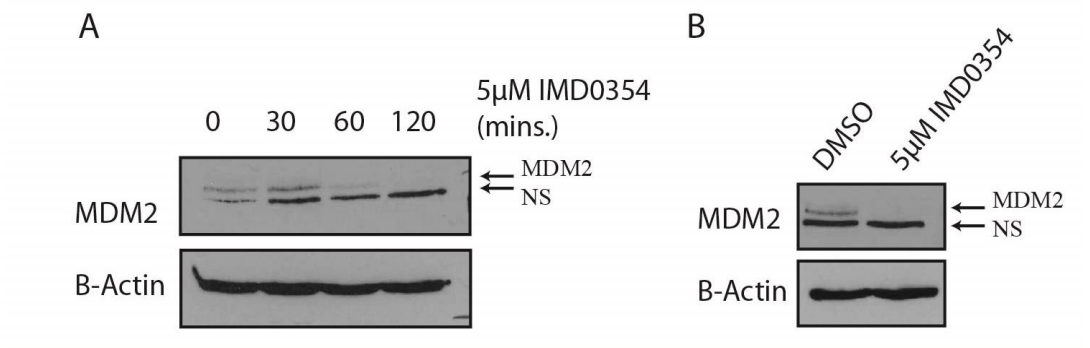

Fig 5.3. Western blotting confirms IMD0354 reduces MDM2 in samples used for MS analysis. (A) T47D cells were treated with 5μM IMD0354 for the indicated time. Cell lysates (20μg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody. **(B)** T47D cells labelled using "Heavy" and "Light" media were treated with DMSO or 5μM IMD0354 respectively for 120mins. Cell lysates (20μg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody.

5.2.2 Analysis of proteome changes resulting from IMD0354 using iTRAQ

The first proteomic experiment presented here aimed to identify proteins which are regulated by treatment with IMD0354. This approach utilised iTRAQ analysis and the work flow of this experiment can be seen in Fig 5.4. Briefly, cells are treated with IMD0354 for 0, 30, 60 and 120 mins. Proteins are then extracted by cell lysis and trypsinised to attain tryptic peptides. The peptides in the different samples are then labelled with iTRAQ tags. The four samples were combined and subject to strong cation exchange (SCX) to fractionate the sample and then analysed by tandem mass spectrometry. For full method see Chapter 2.

The iTRAQ sample was then divided into two and half of the sample was analysed on an ABSciex QStar Elite LC MS/MS. The remaining sample was analysed on an ABSciex 5600 LC MS/MS. The numbers of peptides identified and quantitated are summarised in Table 5.1. The ABSciex Qstar Elite detected 2692 proteins (with an unused score ≥ 1.3) with a low false discovery rate of 0.07%; 2654 of these proteins had sufficient data to quantify. The ABSciex 5600 detected slightly fewer proteins with a total of 2323 (with an unused score ≥1.3) with a false discovery rate of 0.04%; 2276 proteins had sufficient data to quantify. All data is expressed as a ratio using the zero minute time point as the subject. Up-regulated and down-regulated proteins were defined as having ratios of <0.83 or >1.20 respectively with a P-Value <0.05. The highest up and down regulated proteins are detailed in Tables 5.2 and 5.3. Full details of up and down regulated proteins are shown in Supplementary Data (Tables S2-S4).

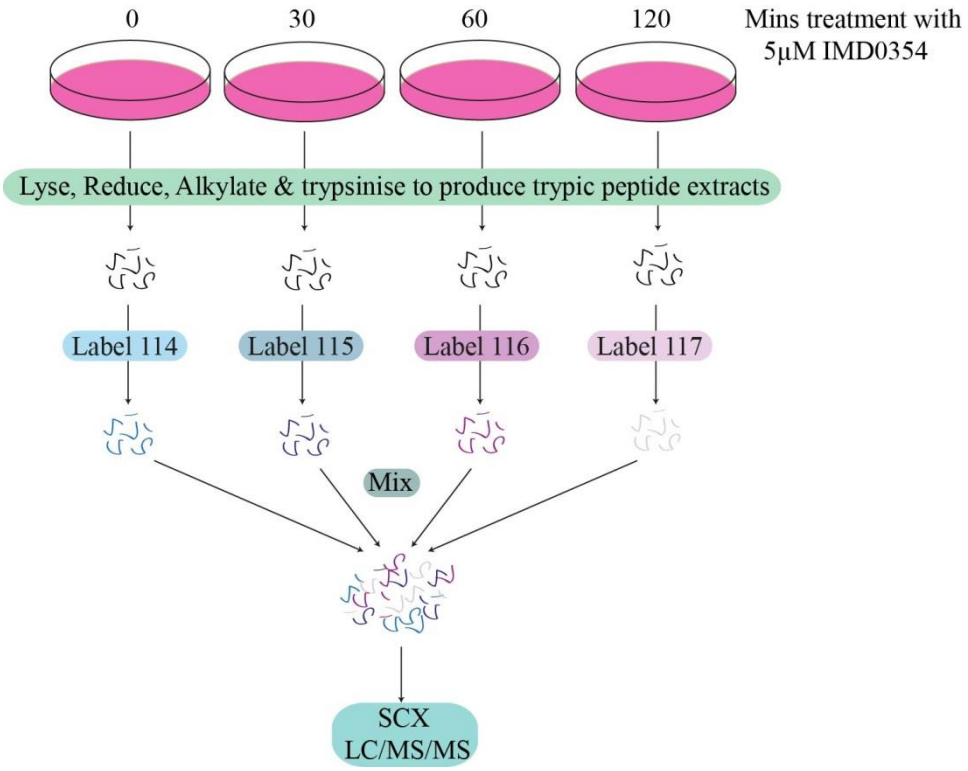

Fig 5.4 iTRAQ proteomics experiment workflow. T47D cells were treated with 5µM IMD0354 for the indicated time. Samples were then lysed to extract soluble proteins. One hundred micrograms of each sample were then reduced, alkylated and subsequently trypsinised overnight. Each sample was incubated with the iTRAQ tags indicated in order to label peptides. The four samples were then combined and fractionated by SCX. Finally samples were analysed by LC/MS/MS.

Table 5.1 Peptide data from iTRAQ proteome experiment.

Instrument	Proteins detected	Total peptides detected	# Reversed protein IDs	False discovery rate
ABSciex QStar Elite	2692	16398	2	0.07%
ABSciex 5600	2323	13482	1	0.04%

Table 5.2 Top 5 up/down regulated proteins after IMD0354 treatment. Data from iTRAQ proteomics experiment (5600 data set with corresponding Qstar values.)

Accession	Symbol	Name	Instrument	% Cov.	Peptides	30:0	P value	60:0	P value	120:0	P Value
P07910	HNRPC	Heterogeneous nuclear ribonucleoproteins C1/C2	5600	42.81	17	0.46	<0.01	1.10	0.27	0.93	0.43
			Qstar	35.95	13	0.70	<0.01	1.00	0.98	0.94	0.45
P05114	HMGN1	Non-histone chromosomal protein HMG-14	5600	43.00	9	0.46	<0.01	1.27	0.10	1.13	0.25
			Qstar	43.00	6	0.64	0.02	0.96	0.68	0.94	0.62
P62805	H4	Histone H4	5600	51.46	26	0.51	<0.01	1.29	0.11	0.95	0.67
			Qstar	63.11	28	0.66	<0.01	1.01	0.90	0.92	0.43
P22626	ROA2	Heterogeneous nuclear ribonucleoproteins A2/B1	5600	54.96	45	0.53	<0.01	0.98	0.81	0.83	0.04
			Qstar	56.37	28	0.69	<0.01	0.93	0.16	0.90	0.04
P14866	HNRPL	Heterogeneous nuclear ribonucleoprotein L	5600	24.62	13	0.58	<0.01	1.01	0.91	0.88	0.21
			Qstar	29.88	15	0.81	<0.01	1.01	0.81	0.94	0.23
P62277	RS13	40S ribosomal protein S13	5600	22.52	6	1.34	0.02	1.31	0.02	1.62	0.02
			Qstar	35.10	7	1.19	0.09	1.22	0.08	1.29	0.10
P39019	RS19	40S ribosomal protein S19	5600	48.28	8	1.39	0.01	1.32	0.02	1.46	0.01
			Qstar	42.80	9	1.09	0.16	1.11	0.10	1.14	0.06
P06733	ENOA	Alpha-enolase	5600	50.69	30	1.43	<0.01	1.30	<0.01	1.27	<0.01
			Qstar	55.80	37	1.09	0.10	1.04	0.34	1.06	0.15
P20700	LMNB1	Lamin-B1	5600	11.09	7	0.72	<0.01	1.43	0.01	1.29	0.01
			Qstar	20.48	10	0.75	0.03	1.34	<0.01	1.21	<0.01
P49207	RL34	60S ribosomal protein L34	5600	35.90	5	1.07	0.41	1.24	0.05	1.43	0.01
			Qstar	28.20	4	0.91	0.20	1.15	0.20	1.32	0.07

Table 5.3 Top 5 up/down regulated proteins after IMD0354 treatment. Data from iTRAQ proteomics experiment (QStar data set with corresponding 5600 values.)

Accession	Symbol	Name	Instrument	% Cov	Peptides	30:0	P Value	60:0	P Value	120:0	P Value
P38159	RBMX	Heterogeneous nuclear ribonucleoprotein G	Qstar	28.13	9	0.63	<0.01	0.91	0.20	0.82	0.01
			5600	28.39	12	0.58	<0.01	1.01	0.70	0.87	<0.01
P05114	HMGN1	Non-histone chromosomal protein HMG-14	Qstar	43.00	6	0.64	0.02	0.96	0.68	0.94	0.62
			5600	43.00	9	0.46	<0.01	1.27	0.10	1.13	0.25
Q9UKV3	ACIN1	Apoptotic chromatin condensation inducer 1	Qstar	9.77	11	0.64	<0.01	0.88	0.07	0.87	0.04
			5600	8.95	13	0.64	<0.01	0.92	0.14	0.87	0.11
P62805	H4	Histone H4	Qstar	63.11	28	0.66	<0.01	1.01	0.90	0.92	0.43
			5600	51.46	26	0.51	<0.01	1.29	0.11	0.95	0.67
P07919	UQCRH	Cytochrome b-c1 complex subunit 6, mitochondrial	Qstar	39.56	3	0.67	0.04	1.29	0.09	1.24	0.12
			5600	16.48	2	0.84	0.28	1.09	0.42	1.15	0.29
P21266	GSTM3	Glutathione S-transferase Mu 3	Qstar	42.67	11	1.35	<0.01	1.30	0.01	1.23	0.03
			5600	12.89	3	1.38	0.12	1.35	0.05	1.28	0.07
P20700	LMNB1	Lamin-B1	Qstar	20.48	10	0.75	0.03	1.34	<0.01	1.21	<0.01
			5600	11.09	7	0.72	<0.01	1.43	0.01	1.29	0.01
P49720	PSMB3	Proteasome subunit beta type-3	Qstar	45.85	8	1.27	0.01	1.13	0.15	1.23	0.06
			5600	21.46	3	1.30	0.09	1.05	0.68	1.17	0.21
P46781	RPS9	40S ribosomal protein S9	Qstar	32.47	9	1.26	0.02	1.20	0.01	1.25	0.02
			5600	20.62	6	1.19	0.18	1.14	0.40	1.23	0.12
P62195	PSMC5	26S protease regulatory subunit 8	Qstar	28.57	10	1.26	0.02	1.19	0.05	1.23	0.08
			5600	22.91	6	1.23	0.04	1.17	0.09	1.11	0.22

5.2.3 Analysis of phosphoproteome changes resulting from IMD0354 using SILAC

The second experiment detailed here attempted to analyse changes to the phosphoproteome resulting from IMD0354 treatment using SILAC labelled cells. For SILAC analysis cells were labelled by growing in "heavy" or "light" media for 6 passages prior to treatment with IMD0354. The "light" media used was control SILAC DMEM media containing unlabelled arginine and lysine amino acids (R0K0), whereas "heavy" media was SILAC DMEM media containing ^{13}C and ^{15}N labelled arginine, and ^{13}C and ^{15}N labelled lysine (R10K8). As IKKβ is a kinase, its inhibition would be expected to result in a reduction in phosphorylation of its substrates as well as further downstream components. This SILAC analysis aimed to identify potential substrates of IKKβ important in mediating the effects of treatment of IMD0354 in T47D cells. The workflow for the SILAC analysis is detailed in Fig 5.5 Full methods are detailed in Chapter 2. Briefly, cells were labelled with heavy and light media and then treated with IMD0354 or DMSO for 2hrs. Samples were then subject to processing including trypsinisation and purification. Phosphopeptides were then extracted by TiO_2 enrichment and samples were again purified. Once samples were processed they were analysed on an ABSciex QStar Elite Hybrid LC MS/MS.

This approach led to the identification of 108 unique peptides (with an unused score of ≥1.3), 90 of which were quantified and represented a total of 63 proteins. The false discovery rate was calculated based on the number of reversed protein IDs identified; as no reversed proteins were identified in this analysis the false discovery rate was 0%. It is likely that this represents a reasonably low false discovery rate combined with few protein identifications. Again, up-regulated and down-regulated phosphopeptides were defined as having ratios of <0.83 or >1.20 respectively. Nineteen phosphopeptides were identified as down-regulated whereas twenty-six phosphopeptides were identified as up-regulated. The highest up and down-regulated phosphopeptides are detailed in Table 5.5 and 5.6. A large proportion of peptides appeared to have missed cleavage sites and further analysis

revealed that the efficiency of tryptic digestion was poor and only 49.7% of peptides did not have one or more missed cleavage sites.

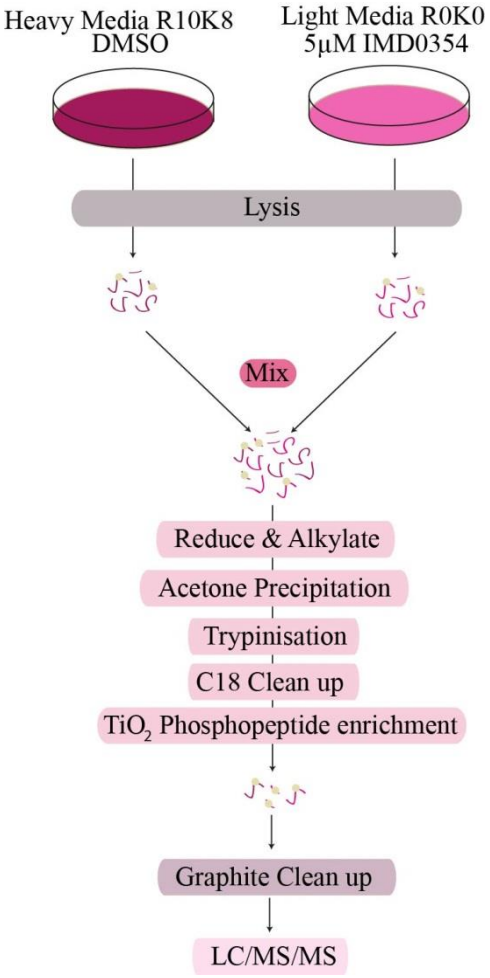

Fig 5.5. Work flow for SILAC phosphoproteomics experiment. Samples are labelled with heavy and light media prior to treatment with DMSO or IMD0354. Samples were lysed and immediately combined. Samples were then reduce and alkylated. Proteins were precipitated using acetone and subsequently trypsinised, Samples were then cleaned on C18 columns and phosphopeptides were extracted using TiO_2. Phosphopeptides were further purified on graphite columns and analysed by LC/MS/MS.

Table 5.4. SILAC phosphopeptides experiment statistics.

Instrument	Peptides detected	Peptides quantified	Proteins quantified	False discovery rate
ABSciex QStar Elite	108	90	63	0%

Table 5.5 Top 5 Down-regulated Phosphopeptides after IMD0354 treatment.

Accession	Symbol	Names	% Cov.	Sequence	Modifications	Cleavages	H:L	% Error
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	1.61	GHYEVTGSDDETGKLQGSGVSLASK	Phospho(S)@8	missed K-L	0.45	5.58
Q9P206	K1522	Uncharacterized protein KIAA1522	1.55	ASPVPAPSSGLHAAVR	Phospho(S)@2		0.57	4.01
Q9NYF8	BCLF1	Bcl-2-associated transcription factor 1	4.13	YSPSQNSPIHHIPSR	Phospho(S)@2; Phospho(H)@10		0.59	1.95
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	7.50	KVEEEQEADEEDVSEEEAESK	Phospho(S)@14	missed K-V	0.60	2.18
Q13247	SFRS6	Splicing factor, arginine/serine-rich 6	3.20	SNSPLPVPPSK	Phospho(S)@3		0.60	3.53

Table 5.6 Top 5 Up-regulated Phosphopeptides after IMD0354 treatment.

Accession	Symbol	Names	% Cov.	Sequence	Modifications	Cleavages	H:L	% Error
P27816	MAP4	Microtubule-associated protein 4	1.21	DMESPTKLDVTLAK	Phospho(S)@4	missed K-L	2.09	4.09
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	1.61	LKSEDGVEGDLGETQSR	Phospho(S)@3	missed K-S	2.09	3.37
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	1.61	SSKASLGSLEGEAEAEASSPK	Phospho(S)@2; Phospho(S)@8	missed K-A	2.07	4.19
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	1.61	SSKASLGSLEGEAEAEASSPK	Phospho(S)@5; Phospho(S)@8	missed K-A	2.00	4.14
P46937	YAP1	Yorkie homolog	3.57	QASTDAGTAGALTPQHVR	Phospho(S)@3		1.96	2.36

5.2.4 iTRAQ data analysis

5.2.4.1 Qstar Elite Vs. 5600

Whilst the ABSciex Qstar Elite lead to the identification of more proteins than the ABSciex 5600, the ABSciex 5600 identified more proteins defined as up or down regulated as can be seen in Fig 5.6. The two machines are broadly similar in design and are both quadrupole time of flight mass-spectrometers. The ABSciex 5600 identified 60 up-regulated protein and 88 down-regulated proteins, whereas the ABSciex QStar Elite identified 19 up-regulated proteins and 79 down-regulated proteins.

Whilst this appears to suggest a large number of disagreements between the two instruments, this is not the case. For analysis a strict cut-off value for up and down-regulation has to be selected. This means that when using data from two MS/MS analyses the two values can fall either side of the cut-off. For example, using the ABSciex Qstar Elite, snRNP200 was defined as not changing after 30mins IMD0354 treatment as it had a ratio of 0.836 whereas the ABSciex 5600 generated a ratio of 0.826 and thus was deemed to have changed. However, the two instruments are clearly producing similar results; further examples are shown in Table 5.2 & 5.3 and supplementary Tables S2-S4. Whilst this example is one the most extreme, when analysing all protein hits identified by only one instrument it was noted that the corresponding value on the other instrument showed a non-significant change but in the same direction 96% of the time (139/145).

Whilst the data between the two MS/MS analysis are broadly similar the data from the QStar Elite appears to identify more conservative changes than the 5600. In order to take forward the most promising leads further analysis largely focusses on data from the QStar Elite.

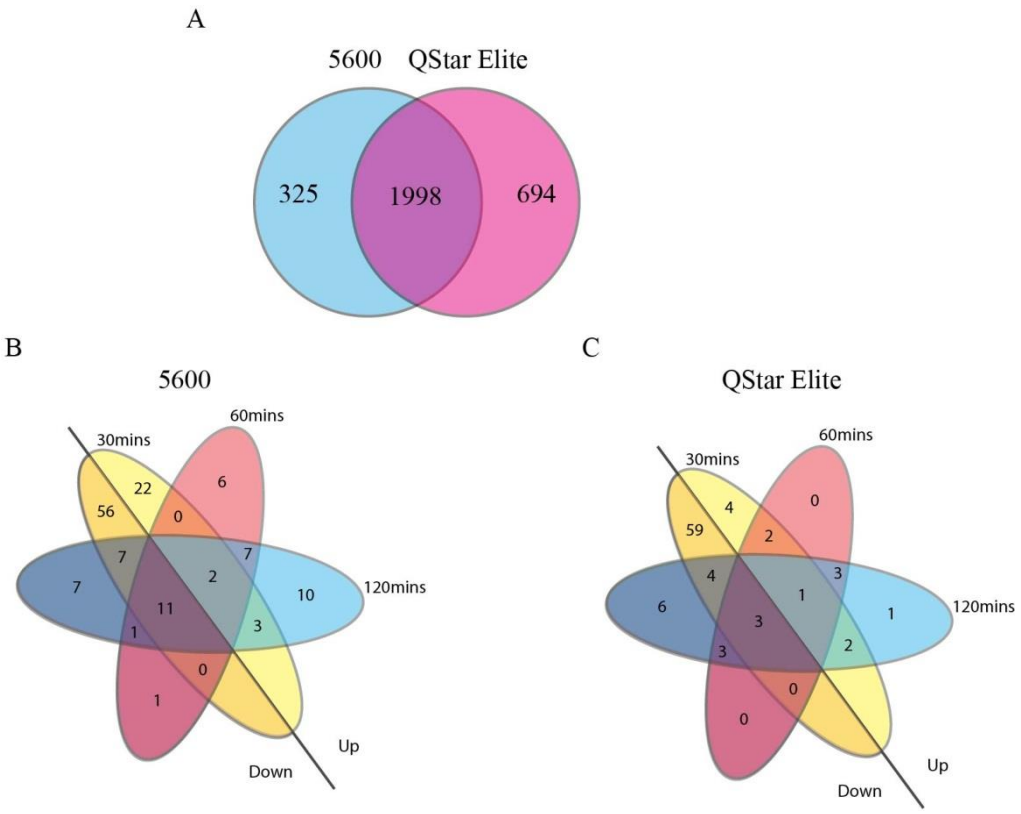

Fig 5.6 Comparison of proteins identified with either the ABSciex 5600 or ABSciex QStar Elite MS instrument in this study. T47D cells treated with 5µM IMD0354 for 0, 30, 60, 120mins and the proteome was analysed by two different MS instuments. MS/MS analysis by the ABSciex Qstar Elite lead to the identification of more proteins than the ABSciex 5600 but identified fewer proteins up/down regulated with IMD0354 treatment. **(A)** Shows the total number of protein identified using the two instuments (ABSciex 5600/ABSciex QStar Elite) as well as the number identified by either instument alone. **(B)** Shows the up and down regulated proteins at each time point in the ABSciex 5600 data set. **(C)** Shows the up and down regulated proteins at each time point in the ABSciex Qstar Elite data set.

5.2.4.2 Pathway analysis

In order to assess the potential interaction between protein hits, a network was created using STRING 9.0 (http://string-db.org/). STRING is a database of known and predicted protein interactions. As can be seen in Fig 5.7, many of the proteins identified in the ABSciex QStar Elite data set as changing following IMD0354 treatment interact with each other and form a large interaction network. This is anticipated as proteins can rarely be altered in isolation from their networks. Observing such changes in networks of interacting proteins supports the validity of each individual result. This network includes a large map with a number of smaller more closely connected groups, as well as some smaller isolated networks. One large group of closely linked proteins which are seen to change in response to IMD0354 treatment are a group of RNA processing and splicing proteins (*e.g.* SF3A3, HnRNPA2B1) which have decreased expression. Smaller interlinked groups include a group of ATP synthase proteins (ATPA1, ATP5B, ATP5H and ATP5J) which are both up and down regulated at different time points and a group of three ribosomal proteins (RPS3, RPS4X and RPS9) which are all up-regulated.

The up-regulated and down-regulated proteins were also analysed for enrichment of pathways, biological process and molecular function represented within the data sets. This was analysed using Panther pathway analysis (http://www.pantherdb.org/pathway/). This software was designed to analyse microarray data but is capable of categorising any protein/gene lists and placing them in functional categories. There was no significant enrichment of any (Panther) pathways found by the software, however four Go biological processes and five Go molecular functions were enriched in the down-regulated data set in comparison to all the identified proteins. These are shown in Fig 5.8 and Fig 5.9. Both analysis types suggest that proteins involved in RNA processing are over-represented in the down-regulated data set. This is consistent with the network analysis showing that an interlinked group of RNA processing and splicing proteins were down-regulated.

As no pathways were seen to be enriched in either the up or downregulated data set a more in-depth analysis was attempted. The entire data set with

corresponding ratio values was analysed to identify subtle changes in pathways regulated by IKKβ inhibition. The data was then analysed using Panther pathway analysis (http://www.pantherdb.org/pathway/). This analysis does not rely on the <0.83 or >1.2 cut-off points and instead uses all the available data. By looking at the pathways as a whole, smaller changes can be identified; greater confidence results from similar changes in a larger group of interlinked proteins. For example a small change in one protein maybe chance but a small change in all the proteins in the same pathway is likely to be more meaningful. The pathways identified by this software are detailed in Table 5.4. and as can be seen, a number of pathways are identified to have changed following IMD0354 treatment. Interestingly, more pathways appear to be up-regulated than down regulated in T47D cells following. Surprisingly the p53 pathway is identified as possibly up-regulated, however, clearly this is unlikely in T47D cells as they only express mutant p53 which does not possess wild-type activity (*274*). Factors involved in Huntington disease are also suggested to be increased, this is of particular interest as IKK has been suggested to be important in the degradation of the normal huntingtin protein (Htt) (*275*). As Huntington's disease is associated with accumulation of an aberrant form of Htt, it seems entirely conceivable that blocking normal degradation of Htt could lead to some changes associated with Huntington's disease. Four adrenergic receptor pathways as well as the adrenaline and noradrenaline biosynthesis pathway are shown to be downregulated in T47D cells, 120 minutes following IMD treatment. This is of particular interest as MDM2 has been implicated in the ubiquitination of a number of proteins within this pathway (*276-278*).

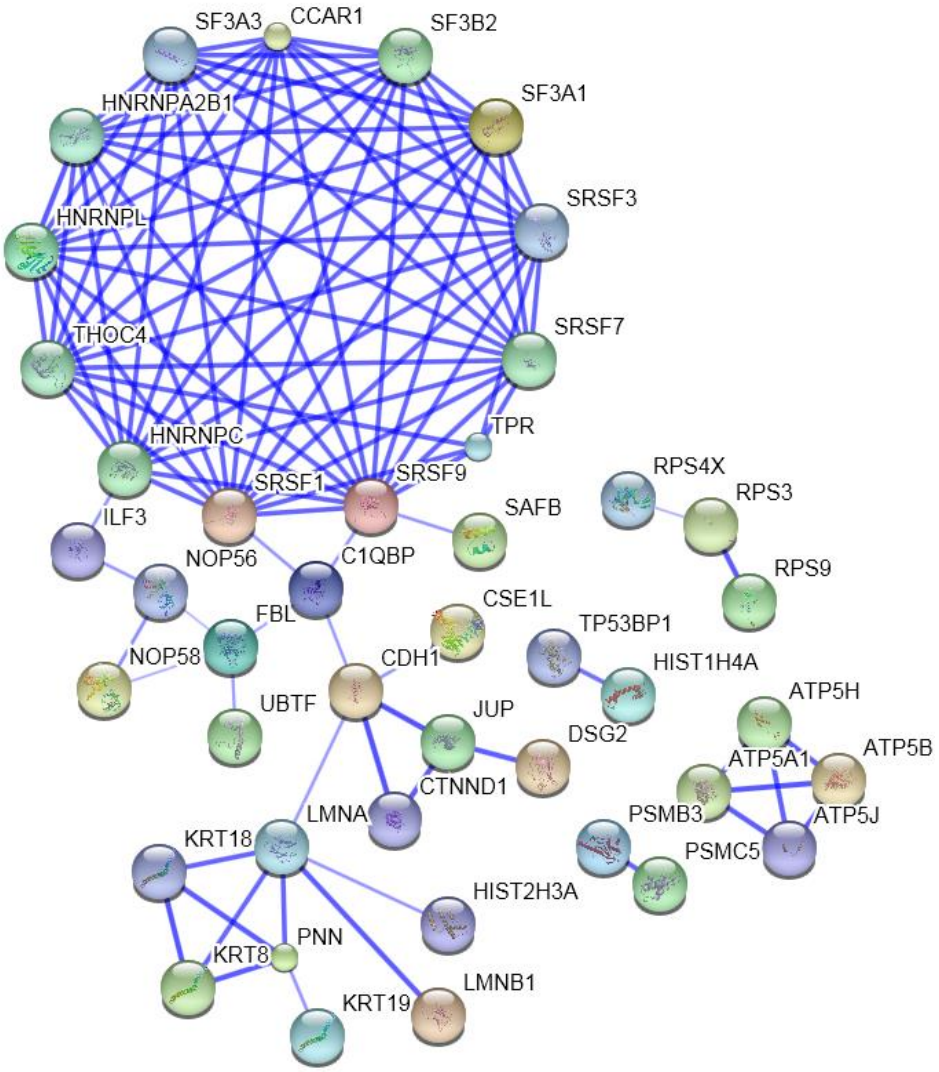

Fig 5.7 Interaction network of proteins identified as changing after IMD0354 treatment (Using data generated by the ABSciex QStar Elite). Interaction network was created on STRING (http://string-db.org/) using the following settings: Active prediction methods – Databases and Experiments, Required confidence –Medium (0.400), View - Confidence view. Thicker lines indicate greater confidence in the interaction.

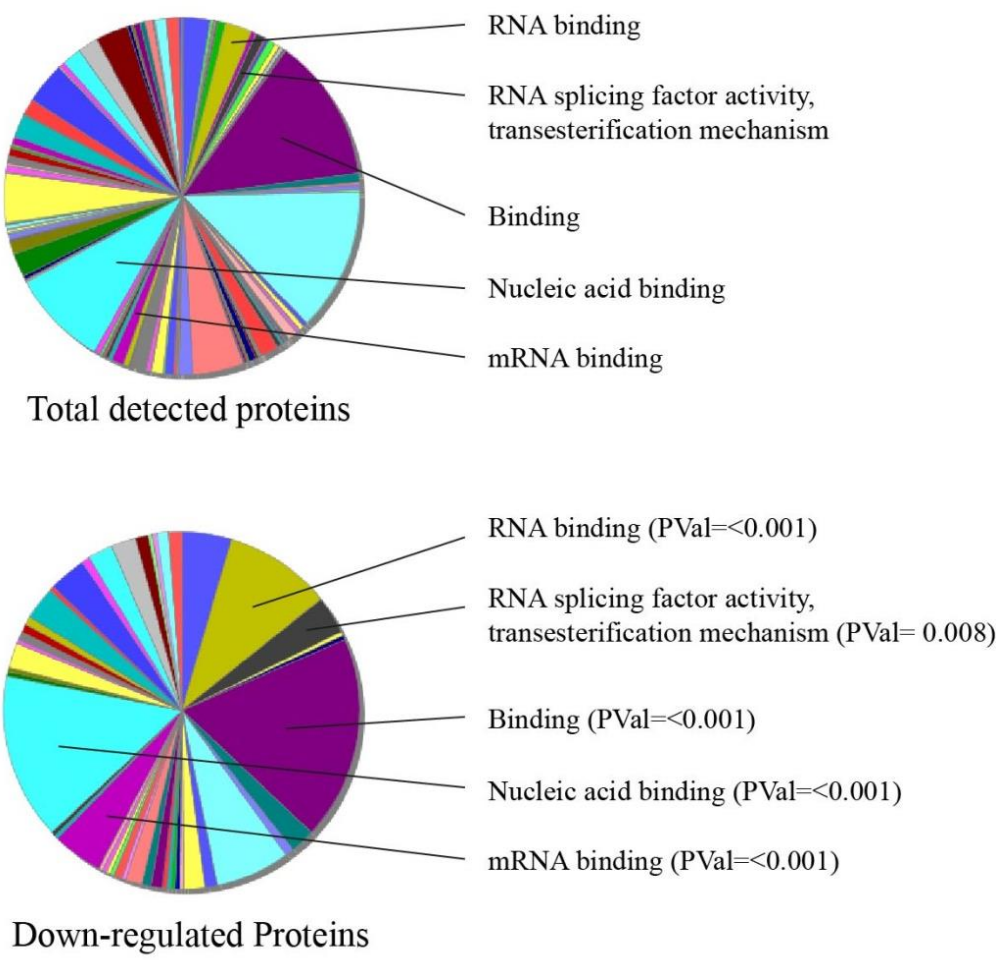

Fig 5.8 The relative abundance of proteins grouped by functions (Go-term) in both total detected proteins and those downregulated proteins identified after IMD0354 treatment. Functional groups which are significantly enriched in the downregulated group (bottom) in comparison to the total data set (top chart) are labelled and P-Values indicated. The corresponding groups are labelled in the total detected proteins group for comparison. No functional groups were seen to be significantly enriched in the up-regulated data set.

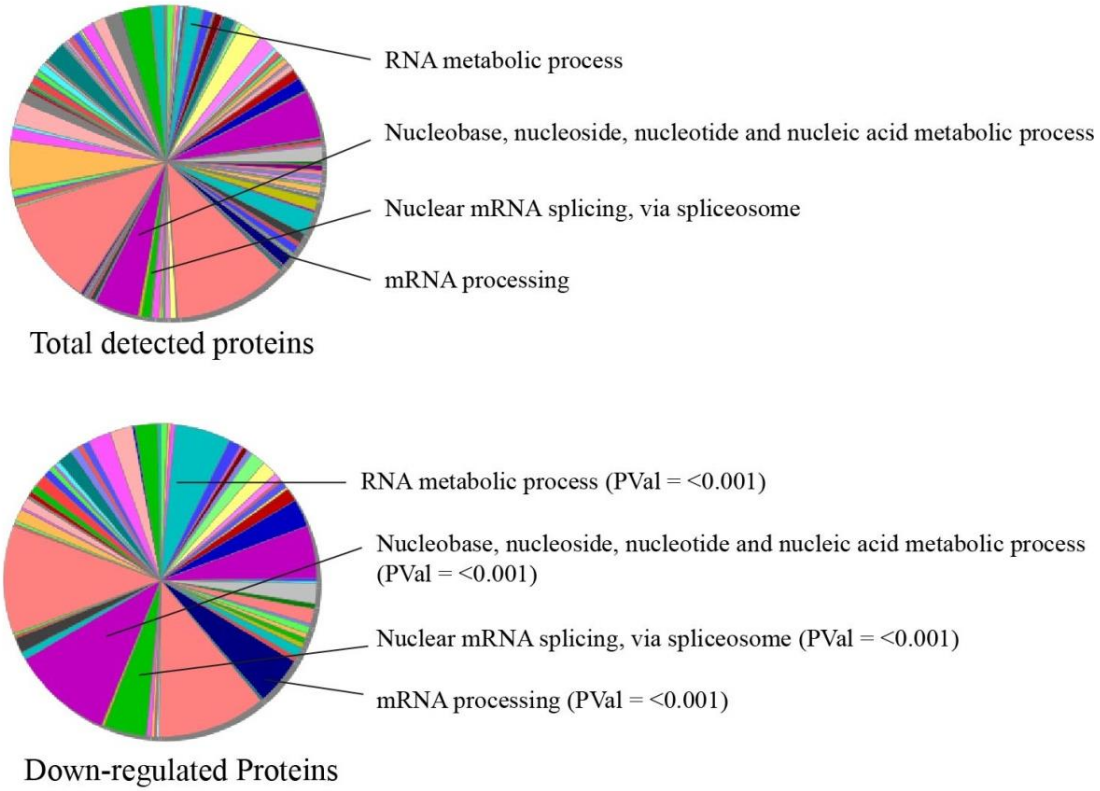

Fig 5.9 The relative abundance of proteins grouped by process (Go-term) in both total detected proteins and those downregulated proteins identified after IMD0354 treatment. Groups which are significantly enriched in the downregulated group (bottom) in comparison to the total data set (top chart) are labelled and P-Values indicated. The corresponding groups are labelled in the total detected proteins group for comparison. No functional groups were seen to be significantly enriched in the up-regulated data set.

Table 5.7 Pathways identified as potentially altered after IMD0354 treatment by Panther pathway analysis. All proteins as well as the ratio fold change after IMD0354 treatment identified from mass analysis were submitted to panther pathway analysis. (http://www.pantherdb.org/pathway/). Detailed are all pathway identified to be significantly altered by this analysis (PVal>0.05). ↑ Indicates positive regulation of the pathway, ↓ indicates negative regulation of the pathway, – indicates no change.

Pathway	30 mins	60 mins	120mins
Ubiquitin proteasome pathway	↑	↑	↑
Angiogenesis	↑	↑	↑
De novo purine biosynthesis	↑	↑	↑
EGF receptor signalling pathway	↑	↑	↑
Parkinson disease	↑	↑	↑
ATP synthesis	↓	-	-
FGF signalling pathway	↑	↑	↑
Glycolysis	↑	↑	↑
Ras Pathway	↑	↑	↑
PDGF pathway	↑	↑	↑
Vitamin D metabolism and pathway	↓	↓	↓
Interleukin signalling pathway	↑	↑	↑
De novo pyrimidine deoxyribonucleotide biosynthesis	↑	↑	↑
p53 pathway glucose deprivation	↑	-	-
p53 pathway	↑	↑	-
Axon guidance mediated by nectrin	↓	-	-
PI3 kinase pathway	↑	↑	↑
Mannose metabolism	↑	-	-
Cytoskeletal regulation by Rho GTPase	↑	-	-
mRNA splicing	↓	-	-
Huntington disease	↑	↑	↑
FAS signalling pathway	-	↑	↑
De novo pyrmidine ribonucleotides biosynthesis	-	↑	↑

T cell activation	-	↑	↑
Purine metabolism	-	↑	-
Axon guidance by semaphorins	-	↑	↑
Beta1 adrenergic receptor signalling pathway	-	-	↓
Beta2 adrenergic receptor signalling pathway	-	-	↓
Alpha adrenergic receptor signalling pathway	-	-	↓
Thyrotropin-releasing hormone receptor signalling pathway	-	-	↓
Oxytocin receptor mediated signalling pathway	-	-	↓
5HT2 type receptor mediated signalling pathway	-	-	↓
Wnt signalling pathway	-	-	↓
Cortocotropin releasing factor receptor signalling pathway	-	-	↓
Beta3 adrenergic receptor signalling pathway	-	-	↓
5HT4 type receptor mediated signalling pathway	-	-	↓
5HT1 type receptor mediated signalling pathway	-	-	↓
Adrenaline and noadrenaline biosynthses	-	-	↓
Cadherin signalling pathway	-	-	↓

5.2.5 SILAC data analysis

The phosphorylation status of peptides identified in this experiment were again deemed to be up or down regulated if the ratio to control was <0.83 or >1.2. This led to the identification of relatively few phosphopeptides "hits". However, the proportion of phosphopeptides deemed to have changed as a result of IMD0354 treatment are a large proportion of the total peptides identified (43/90=48%). This is a surprising result as such large scale changes to the phosphoproteome would not be anticipated so rapidly after a specific kinase inhibitor. The small number of phosphopeptides identified rules out a number of types of analysis; i.e. pathway analysis which relies on a large dataset to be successful. Interestingly, one of the phosphopeptides hits identified by SILAC analysis was a cAMP-dependent protein kinase type II-alpha regulatory subunit which is known to be important in G-Protein coupled receptor (GPCR) pathways including adrenergic pathways, adding confidence to the idea that this pathway may be affected by IMD0354.

The SILAC experiment was initially intended to identify potential substrates of IKKβ in un-stimulated T47D cells. IKKβ are known to phosphorylate substrates with a consensus motif of DSΨXXS/T (*279*)(Fig 5.10) were Ψ indicates a hydrophobic residue, X indicates any residue and S/T indicates a serine or a threonine. Therefore, phosphopeptides hits were analysed for the presence of this motif. Unfortunately peptides hits with this motif were not identified. This is not entirely unanticipated, as IKKβ has also been shown to phosphorylate a number of proteins without the DSΨXXS peptide motif (*280, 281*).

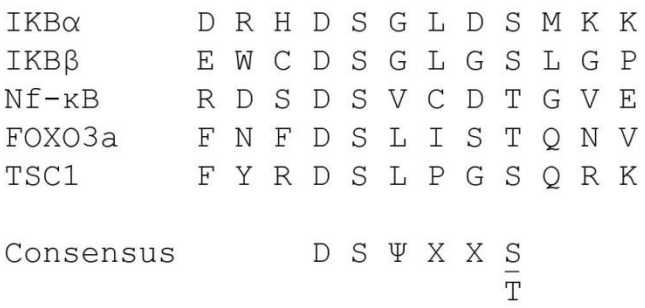

Fig 5.10 Consensus motif of IKKβ substrates (Figure adapted from Kuo et al. (2009) (*279*)**.** Ψ indicates a hydrophobic amino acid; X indicates any amino acid.

5.2.6 Preliminary validation

Proteomic analysis, such as the data presented here, gives large quantities of data that needs to be processed. Initial analysis of proteome changes resulting from IMD0354 treatment lead to the identification of a total of 96 proteins identified with the ABSciex QStar Elite data set and analysis of the phosphoproteome using SILAC led to the identification of 31 proteins with altered phosphorylation states. The proteins identified to have changed may well represent proteins which have been altered by IMD0354. However in this pilot study the mass spectrometry data only represents a single replicate, therefore, further analysis is needed to validate these results. However, clearly there are too many proteins to validate them all. There are various methods of analysis available aimed at trying to select the most interesting or relevant data from a large data set. Two different approaches are presented here. The first utilises our own knowledge to select data which fits in with what we know about MDM2 and aimed to investigate one of the potentially important pathways revealed by the data analysis. Specifically, the effect of IMD0354 on the β-adrenergic pathway and proteins involved in GPCR pathways were investigated by western blot (in collaboration with George Baillie and Krishna Yalla, University of Glasgow). The second approach specifically tried to identify those proteins which are likely to be mediators of the reduction of MDM2 protein expression and utilised an empirical approach. Seventy five protein hits were selected from iTRAQ and SILAC experiments, these targets were then knocked-down in T47D cells using siRNA to determine what effect, if any, there was on MDM2 protein levels.

5.2.6.1 β-adrenergic and β-arrestin pathway.

The potential down-regulation of the β-adrenergic pathway in response to IMD0354 treatment was of particular interest due to the fact that MDM2 has been shown to be an important factor within this pathway (*277, 278, 282*). One component of this pathway is β-arrestin, which is important in GPCR turnover and desensitisation. β-arrestin is ubiquitinated by MDM2 after GPCR stimulation and this

ubiquitination is required for the internalisation of GPCR (*282*). β-arrestin oligomers have also been shown to control MDM2 localisation and promote MDM2 nuclear export (*278*). Furthermore MDM2 is also required for the ubiquitination of G-protein coupled receptor kinase 2 (GRK2), which promotes GRK2 degradation and is also dependent upon β-arrestin. GRK2 phosphorylates GPCRs after agonist stimulation and plays a role in receptor desensitisation (*277*).

In order to assess the effect of IMD0354 on the β-adrenergic pathway, the effects of IMD0354 treatment on a number of proteins within this pathway were assessed. The effect of IMD0354 treatment on PKA subunits C and R1α, β-arrestin and phospho-β2-adrenergic receptor were assessed by Krishna Yalla (University of Glasgow). As can be seen in fig 5.11 IMD0354 treatment does not appear to lead to any substantial changes in the levels of any of these proteins within the β adrenergic cascade. It was initially intended to assess total β2-adrenergic receptors levels as well as phospho-β2-adrenergic receptors levels however unfortunately we were unable to identify antibodies which could successfully detect total protein in this cell line. As no other changes in the pathway were detected this was not pursued further.

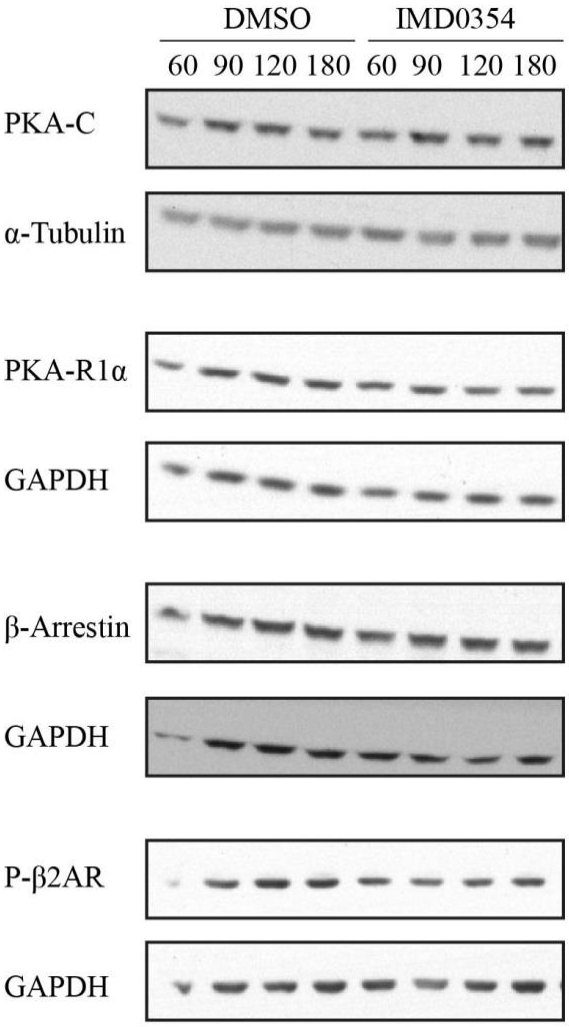

Fig 5.11. β2-adrenergic receptor pathway components remain unaltered by IMD0354 treatment. T47D cells were treated with DMSO or IMD0354 for the indicated time. Cell lysates were then assessed by western blotting to determine relative levels of PKA-C, PKA-R1α, β-arrestin and phospho-β2-adrenergic receptor using the anti-PKA-C, anti-PKA-R1α, anti-β-arrestin and anti-phospho-β2-adrenergic receptor antibodies. Equal loading was also assessed using anti-Tubulin/anti-GAPDH antibodies. (Cell pellets sent to Krishna Yalla (University of Glasgow), who completed the western blotting).

5.2.6.2 siRNA screen

An siRNA screen was set up to identify mediators of MDM2 stability; 75 hit proteins were selected from the QStar Elite iTRAQ data set and the SILAC data set for analysis. The phosphoproteome experiment was a more complex procedure which identified relatively few peptides and the experiment suffered from poor trypsinisation efficiency, therefore it was considered that the confidence in the iTRAQ data set was higher. As such, most of the 75 targets selected were selected from the iTRAQ data set. For details of all targets selected and targeting siRNA for each see Supplementary Data (Table S7).

A siRNA treatment of 40nmol for 48hrs was selected as experience within the lab suggests that this is a concentration and timescale which should lead to a reduction of most targets (J. O'Donoghue and A-S. Huart, Personal Communication). In addition, this treatment time and siRNA concentration was shown to successfully reduce MDM2 in Fig 4.5A. Initially, the siRNA screen was conducted without IMD0354 treatment. Specifically, the screen aimed to identify up-regulated protein hits whose siRNA knockdown increased MDM2 protein expression and down-regulated hits whose siRNA knockdown decreased MDM2 levels. This strategy is summarised in Fig 5.12. The reason for this is assuming that these hits identified by MS are upstream of MDM2 then an up-regulated protein ought to be a negative regulator of MDM2 stability whereas a down-regulated protein ought to be a positive regulator of MDM2 stability. When analysing the SILAC phosphopeptide hits, a change in MDM2 levels in either direction was considered. This is because the effect of changes in phosphorylation status of hit proteins on its activity is not certain.

By utilising this approach, only one hit lead to the expected effect on MDM2 in the desired direction. As can be seen in Fig 5.13A, siRNA targeting RPS3, which was shown to be up-regulated by IMD0354 in MS/MS analysis, lead to a subtle increase in MDM2 levels. This is particularly interesting as RPS3 has been shown to interact with MDM2 and additionally has been suggested to be a component of the Nf-κB pathway (*279, 283*).

Next, siRNAs targeting up-regulated MS hits were also screened with the addition of IMD0354 for those which could rescue the reduction in MDM2 protein. T47D cells were treated with 40nM siRNA for 48Hrs followed by 5μM IMD0354 for 2Hrs. Three targets were identified which appeared to be able to rescue the loss of MDM2 expression which were RPS3, RPS4X and RPS9 (Shown in Fig 5.13B). These proteins are all ribosomal subunit proteins. However many ribosomal proteins, including RPS3 and RPS4X, have recently been revealed to additionally have non-ribosomal functions (*284*). Interesting all three of these proteins are known to interact with one another (As shown in Fig 5.7).

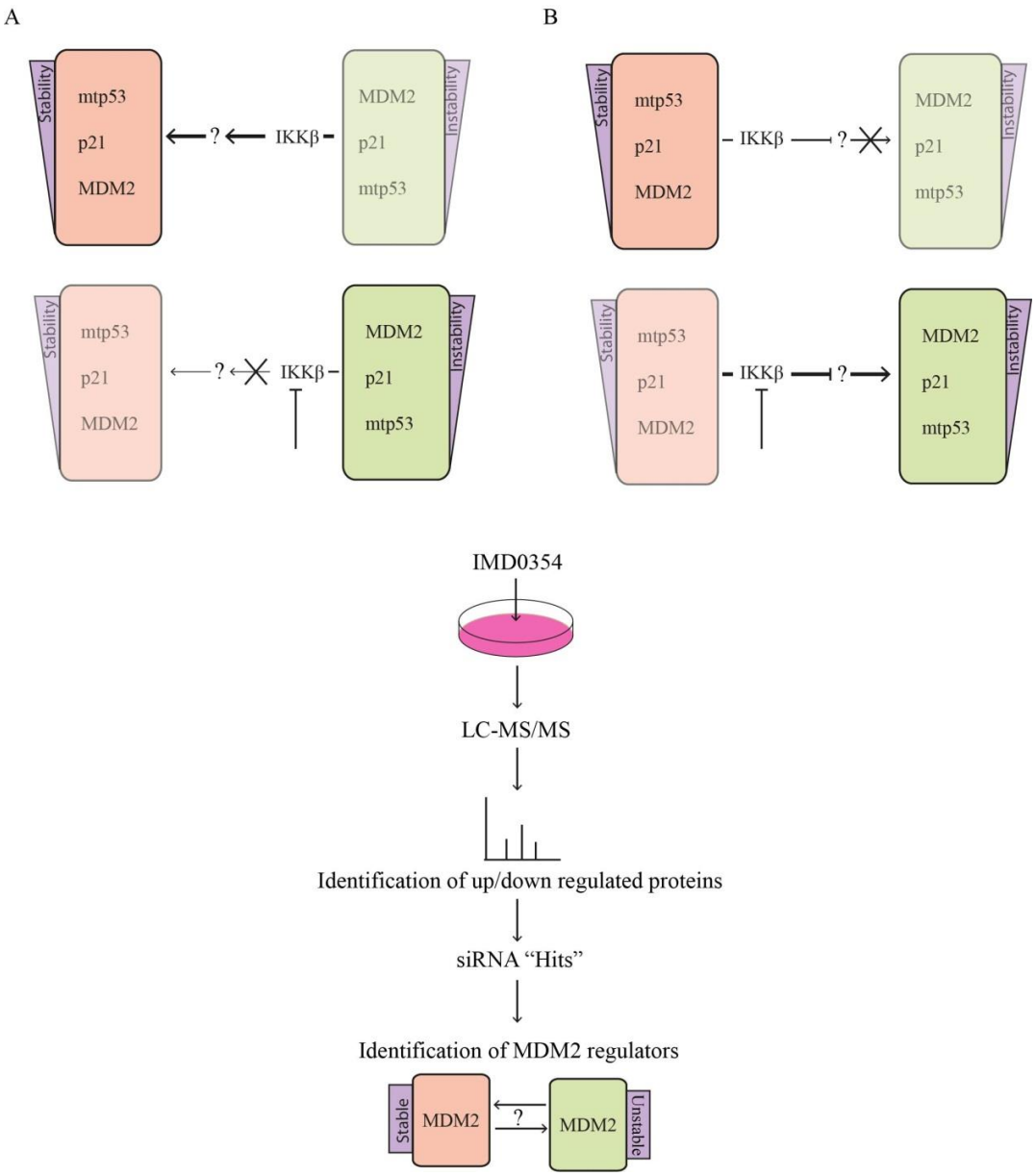

Fig 5.12 Schematics of potential mechanisms by which IKKβ could control MDM2, mtp53 and p21 protein levels; (A) IKKβ may promote a factor(s) which enhances the stability of proteins including MDM2, p21 and p53. Inhibition of IKKβ would reduce this factor(s) and reduce levels of MDM2, p21 and p53. Alternatively **(B)** IKKβ may down-regulate a factor(s) which reduces the stability of proteins including MDM2, p21 and p53. Inhibition of IKKβ would allow this factor(s) to increase and reduce levels of MDM2, p21 and p53. **(C)** shows the workflow of an approach to identify such factors; cells were treated with IMD0354 and subject to tandem MS analysis. This allowed the identification of up and down regulated protein hits. Hit proteins were then screened by siRNA for those which affect MDM2 protein levels.

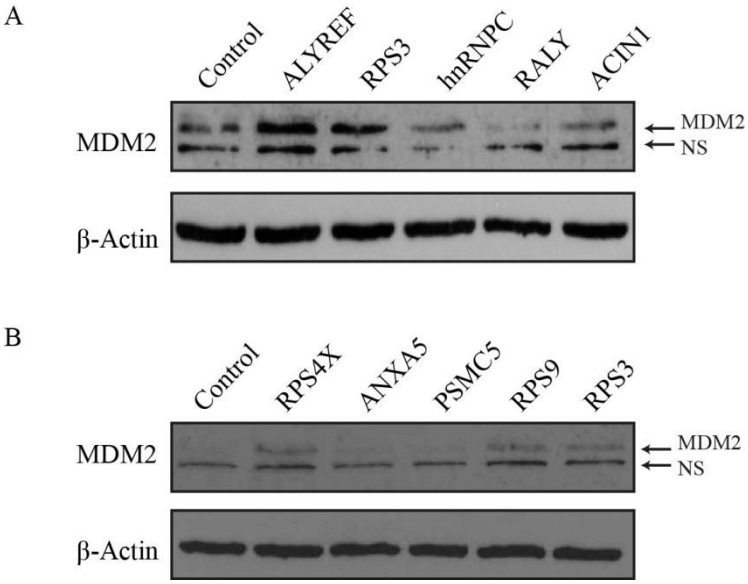

Fig 5.13 RPS3, RPS4X and RPS9 can regulate the levels of MDM2 in cells treated with IMD0354. 75 siRNAs targeting proteins seen to be up/down-regulated or have altered phosphorylation status after IMD0354 treatment were screened for their ability to alter the levels of MDM2 in T47D cells. T47D cells were treated with control or target siRNA 48Hrs prior to harvesting. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody. Examples of 5 results from this experiment along with the non-targeting siRNA control are shown in **(A)**. 19 siRNAs targeting proteins seen to be up-regulated or having altered phosphorylation status after IMD0354 treatment were screened for their ability to rescue levels of MDM2 in T47D cells after IMD0354 treatment. T47D cells were treated with control or target siRNA 48 Hrs prior to harvesting. Cells were additionally treated with 5µM IMD0354 2hrs prior to harvesting. Cell lysates (20µg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody. Examples of 5 results from this experiment along with the non-targeting siRNA control are shown in **(B)**.

5.2.7 The role of RPS3 in the response to IMD0354 treatment

RPS3 siRNA was identified to lead to the up-regulation of MDM2 protein levels both with and without IMD0354, and as RPS3 has previously been seen to interact with proteins within both the p53 and IKK pathways (*281, 283*) it was selected for further analysis. siRNA screening suggests that RPS3 may be important in mediating IMD0354 induced reduction of MDM2 expression. The proteins selected for siRNA screening including the ribosomal proteins; RPS3, RPS4X and RPS9 were selected for analysis as they were seen to be altered by IMD0354 in the ABSciex Qstar Elite iTRAQ results (Table 5.8). The ABSciex Qstar Elite data set was investigated rather than the ABSciex 5600 technical replicate as it had more conservative results. However, retrospective analysis shows that RPS3 was identified as "hits" in the 5600 data set (Table 5.8). RPS4X and RPS9 showed increased levels in the ABSciex 5600 data set at 120mins after IMD0354 treatment (1.21 and 1.23 respectively) but failed to achieve a significant P-value (0.07 and 0.12 respectively). This may have resulted from decreased coverage in this data set compared to the ABSciex QStar Elite data set (for RPS9 6 peptides versus 9 peptides, for RPS4X 7 peptides versus 5). Network analysis of the ABSciex 5600 data set as a whole (Fig 5.14) indicates that a large number of interlinked ribosomal proteins were affected; strengthening the idea that IMD0354 may disrupt the ribosomal proteins.

As can be seen in Fig 5.15A, results showed, in agreement to the results of MS/MS, that overtime IMD0354 leads to the up-regulation of RPS3. Whilst the general pattern of RPS3 levels after IMD0354 treatment remained largely consistent between MS/MS and western blotting, western blotting revealed a much greater level of up regulation of RPS3 overall (Fig 5.16). This is perhaps not surprising as research has shown that iTRAQ MS can underestimate the relative changes occurring (*285*). As a relatively large number of proteins were analysed by siRNA screen, it was not possible to confirm that siRNA was successful with each target and therefore this means that some false negatives may be present in the screen. However, it was confirmed that the treatment with 40nM RPS3 targeting siRNA was sufficient to lead to a reduction in RPS3 protein expression (Fig 5.15B). When

attempting to reproduce the effect of RPS3 siRNA seen in the screen, RPS3 targeting siRNA did not appear to alter MDM2 levels without IMD0354 treatment. However with IMD0354 treatment RPS3 targeting siRNA reduced the loss of MDM2 protein levels by IMD0354 (Fig 5.15C).

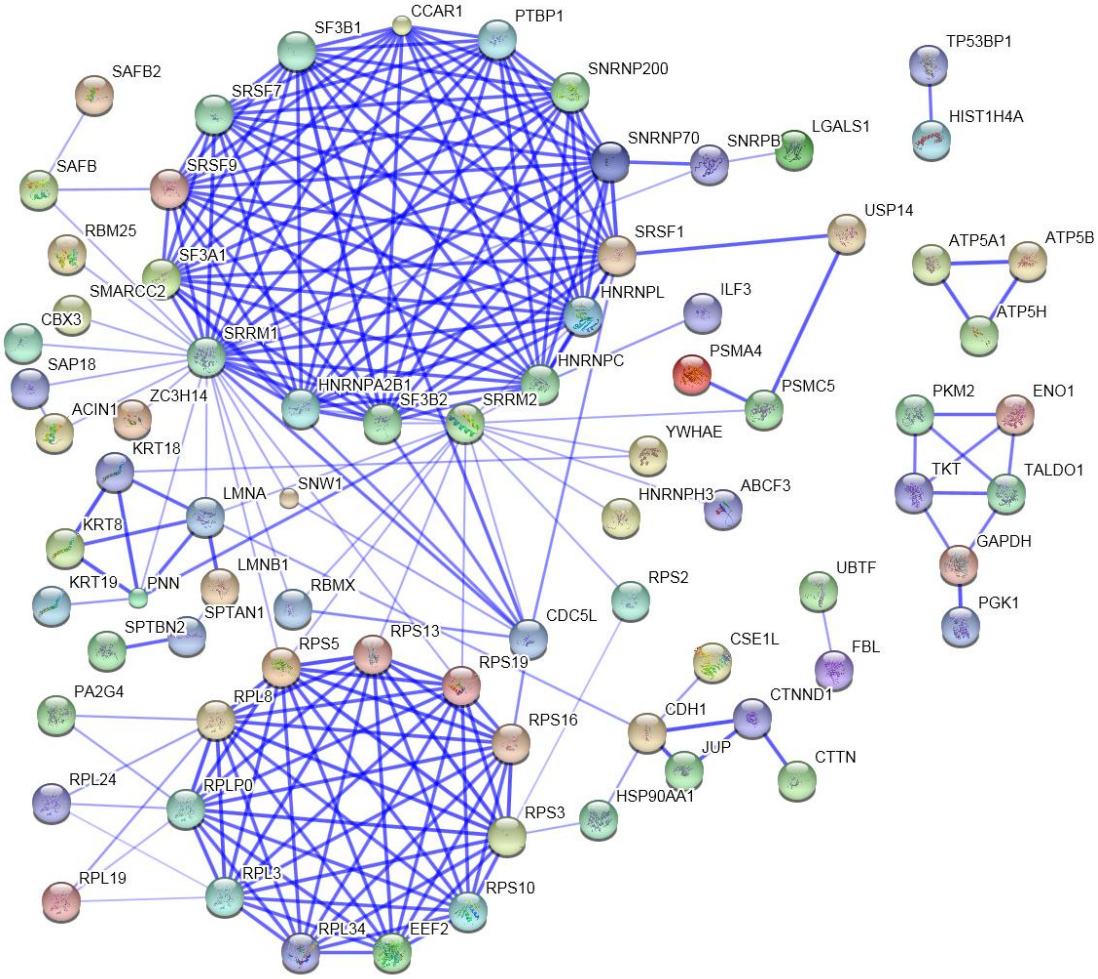

Fig 5.14 Interaction network of proteins identified as changing after IMD0354 treatment (Using data generated by the ABSciex 5600). Interaction network was created on STRING (http://string-db.org/) using the following settings: Active prediction methods – Databases and Experiments, Required confidence –Medium (0.400), View - Confidence view. Thicker lines indicate greater confidence in the interaction.

Table 5.8 Ribosomal proteins identified to be up-regulated after IMD0354 treatment (QStar data set with corresponding data from the 5600)

Accession	Symbol	Name	Instrument	% Cov.	Peptides	30:0	P Value	60:0	P Value	120:0	P Value
P23396	RPS3	40S ribosomal protein S3	Qstar	44.9	14	1.21	0.01	1.04	0.44	1.11	0.21
			5600	24.3	5	1.29	0.03	1.02	0.81	1.08	0.51
P46781	RPS9	40S ribosomal protein S9	Qstar	32.5	9	1.26	0.02	1.20	0.01	1.25	0.02
			5600	20.6	6	1.19	0.18	1.14	0.40	1.23	0.12
P62701	RPS4X	40S ribosomal protein S4, X isoform	Qstar	28.9	7	1.22	0.04	1.20	0.05	1.25	0.02
			5600	19.0	5	1.16	0.12	1.12	0.21	1.21	0.07

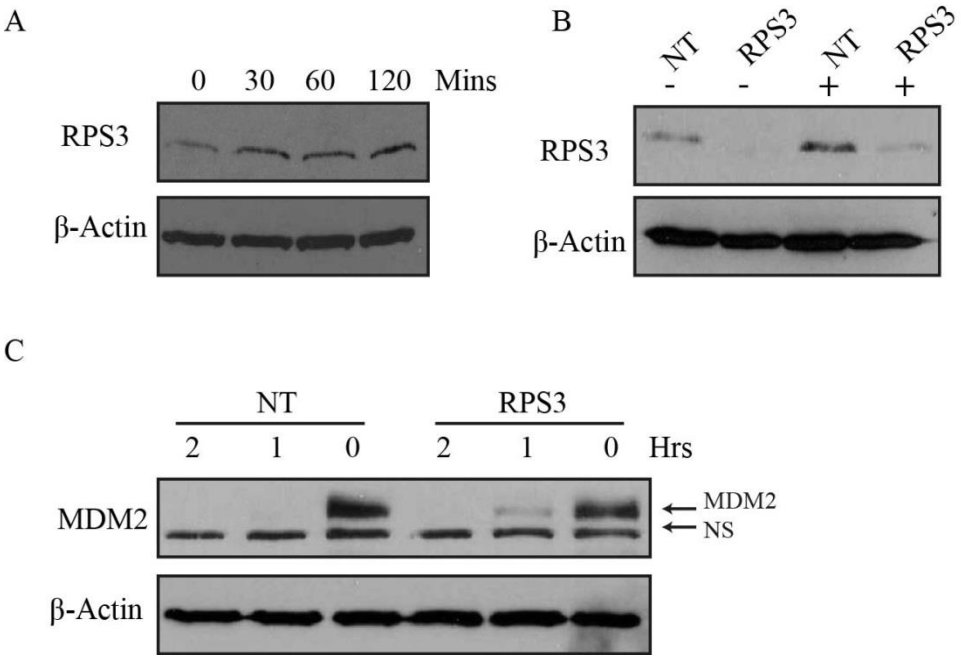

Fig 5.15 Reduction of MDM2 by IMD0354 is partially dependent upon RPS3. (A) T47D cells were treated with 5μM IMD0354 for the indicated time. Cell lysates (20μg/well) were then assessed by western blotting to determine relative RPS3 levels using an anti RPS3 antibody. Equal loading was also assessed using anti-β-actin antibody. **(B)** T47D cells were treated with either non-targeting siRNA (NT) or siRNA targeting RPS3. Cells were then treated with 5μM IMD0354 or DMSO for 2Hrs. Cell lysates (20μg/well) were then assessed by western blotting to determine relative RPS3 levels using an anti RPS3 antibody. Equal loading was also assessed using anti-β-actin antibody. **(C)** T47D cells were treated with either non-targeting siRNA (NT) or siRNA targeting RPS3. Cells were then treated with 5μM IMD0354 for 2Hrs (2), DMSO for 1Hrs and then 5μM IMD0354 for 1Hr (1) or DMSO for 2Hrs (0). Cell lysates (20μg/well) were then assessed by western blotting to determine relative MDM2 levels using the anti-MDM2 antibody, 2A10. Equal loading was also assessed using anti-β-actin antibody.

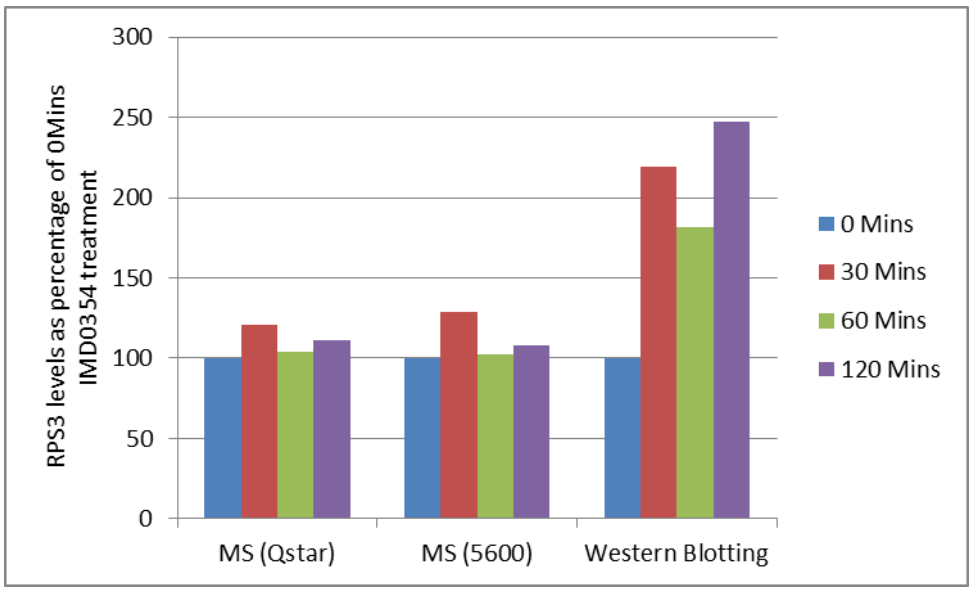

Fig 5.16 Western blotting reveals a greater increase than mass spectrometry in RPS3 induced by IMD0354. Shown are relative levels of RPS3 after IMD0354 treatment at the indicated time-points relative to the 0hrs time-point. Data from western blotting was quantified using ImageJ software and normalised to β-actin levels.

5.3 Discussion

This study used a proteomic approach to investigate proteins up-stream of MDM2 and co-regulated with MDM2, after IMD0354 treatment. Analysis of the whole proteome allows for a much less biased analysis of changes resulting from a specific manipulation or stress. This is due to the fact that, at least initially, the analysis is not driven or limited by our current knowledge.

In this study, 4plex iTRAQ was used to assess proteome changes resulting from treatment of T47D cells with the IKKβ inhibitor IMD0354 over time. This study showed that IMD0354 lead to relatively few changes in the total proteome within the first 2Hrs. This is reassuring as it suggests that inhibition is not leading to mass down-stream changes within the time scale. Interestingly, a large number of hit proteins were seen to be changed at 30 minutes after IMD0354 treatment but returned to control levels by 1hr after treatment. This suggests that IMD0354 may lead to different types of changes with differing dynamics. IMD0354 immediately leads to the transient regulation of a number of proteins as observed by MS/MS. A number of proteins are also subject to a much more sustained change. It is tempting to speculate that this second wave of changes may result from the initially transient wave however this remains to be established.

Network analysis of all proteins seen to have changed following IMD0354 treatment identified two large hubs of proteins. Specifically, it identified a group of proteins involved in mRNA splicing and transport and a second smaller network composed of ribosomal proteins. This is particularly interesting in view of the fact that previous experiments (Fig 4.16 and Fig 4.17) have shown that the down regulation of MDM2 following IMD0354 treatment is likely resulting from alteration in *de novo* protein production. It was shown earlier that MDM2 mRNA levels were reduced by about 40% after 2Hrs of IMD0354 treatment (Fig 4.17). This suggests that mRNA reduction, as well as potentially other mechanisms involved in protein synthesis are important in MDM2 regulation. It was also shown that a change in protein turnover was not involved in MDM2 reduction. The results presented here suggest that a number of proteins involved in mRNA processing and RNA binding are

altered in response to IMD0354 which is consistent with the changes observed in MDM2 mRNA levels. The degree of specificity of such changes is a yet not clear.

In Chapter 4 it was shown that p53 pathway and D-type cyclins are down-regulated as a result of IMD0354 treatment. The mass spectrometry data presented here shows that only a small proportion of proteins are changing in response to IMD0354 treatment. However, if protein synthesis is altered, proteins with faster turnover rates will be lost earlier. Faster turnover rates may be more common in lower abundance proteins which are less likely to be identified by mass spectrometry. Further work will be needed to explore the generality of the effect of IMD0354 on protein synthesis and mRNA processing. It should be noted that many types of cellular stress lead to reduced protein synthesis, however, the synthesis of certain proteins particularly those involved in survival or apoptosis are sustained and others are stabilised by reduced degradation (*286, 287*). Thus, the effect on the p53 pathway under such conditions is important. Furthermore, inhibitors of transcription and/or translation are being explored, or in some cases being used, as cancer therapy as they have been shown to selectively kill cancer cells (*210*). This specificity is thought to be related to the reduction of specific short lived oncogenic proteins to which the cancers are addicted.

Changes in ribosomal proteins may be of particular interest in future studies as screening of siRNAs targeting a number of hit proteins indicated that RPS3 and potentially RPS4X and RPS9 may be important in the IMD0354 mediated loss of MDM2 protein. The proteins that were selected for siRNA screening were proteins identified as changing in the QStar Elite iTRAQ data set. This data set was analysed as it showed more conservative changes than the data from the ABSciex 5600. However, the three ribosomal proteins; RPS3, RPS9 and RPS4X were all identified in both data sets and showed broadly similar patterns of change . RPS3 is a 40S ribosomal subunit although it, like many other ribosomal proteins, also has a number of non-synthesis roles (*281, 284*). A number of ribosomal subunit proteins have been shown to interact with the p53 pathway (*283, 288, 289*). Evidence suggests that ribosomal subunits may be important for coupling ribosome stress to

the p53 apoptotic pathway (*288, 289*), for example, RPL11 has been shown to induce p53 activity. Other evidence suggests that ribosome-p53 pathway interaction is not limited to ribosome stress conditions (*283*). For example, the ribosomal protein RPL26 has been shown to be able to bind p53 mRNA and enhance it translation (*290*), whilst binding of MDM2 to RPL11 repressed this effect (*291*). This effect does not appear to be dependent upon ribosomal stress.

RPS3 has been shown to interact with both MDM2 and p53 and consequently protects p53 from ubiquitination. Yadavilli et al. (2009) showed that siRNA targeting RPS3 leads to a reduction in MDM2 protein levels which appears contradictory to data here (*283*). However, they showed that RPS3 siRNA reduced the levels of MDM2 in response to H_2O_2 treatment and MDM2 levels were broadly similar in untreated cells. Similarly in this study RPS3 siRNA appeared to have a greater influence on the levels of MDM2 in IMD0354 stimulated cells. This indicates the possibility that the effect of RPS3 on MDM2 may depend on pathway stimulation. Yadavilli et al. (2009) also showed that the RPS3 siRNA used lead to a reduction in cytosolic, free RPS3 but did not affect RPS3 bound to ribosomes. Potentially, different siRNA may differentially alter the pools of target protein so care must be taken when comparing siRNA experiments.

To our knowledge RPS4X is not known to directly interact with either p53 or MDM2 however as RPS4X interacts with other ribosomal proteins which are known to interact with both p53 and MDM2 there is the possibility of indirect regulation. Furthermore, the importance of interactions between many ribosomal proteins and the p53 pathway are only just coming to light and clearly there is the possibility that further interactions will be identified in future. RPS9 has however been noted to affect the p53 pathway, Lindström and Nistér (2010) showed that knock-down of RPS9 lead to an activation of the p53 pathway as a result of increased p53 stability which was dependent upon RPS11 (*292*). RPS11 has been shown to increase p53 stability via a negative regulation of MDM2 (*288*).

Whilst it is interesting that ribosomal proteins such as RPS3 and RPS9 can influence both p53 and MDM2 at the protein level, the data here suggests that

changes in MDM2 protein level after IMD0354 treatment occur as a result of changes at a translational or pre-translational level. Research does suggest that RPS3 may play a role in controlling transcription of particular genes has also suggested a link between IKKβ and RPS3. In fact RPS3 has been suggested to be a non-Rel member of the Nf-κB family and can control the transcription of a subset of Nf-κB induced genes (*293*). Furthermore, the activation of RPS3 in this pathway is thought to rely on phosphorylation by IKKβ (*281*). On the face of it therefore, it may seem contradictory that IKKβ inhibition would lead to an increase in RPS3 levels, however it remains undetermined as to whether this RPS3 increase is indicative of increased activity. Interestingly, a number of stimuli that promote the activity of p53 and inhibition of MDM2 via RPL11 activity actually lead to a decrease in the levels of RPL11 (*292*) supporting the idea that protein level is in some cases a poor indicator of activity.

Whilst siRNA targeting of RPS3 lead to reduced loss of MDM2, with IMD0354 treatment this did not represent total recovery of Mdm2 levels to control. In fact, at the longer treatment time of 2hrs, no effect of RPS3 siRNA was observed. As a large number of ribosomal proteins have been shown to bind to MDM2, there is the potential for some redundancy in these roles and may be a point of interest in further studies. This may be supported by the fact that the data presented here suggests as many as 13 other ribosomal proteins may also be altered by IMD0354 and the siRNA screen suggests that RPS4X and RPS9 are also potential regulators of MDM2 within an IMD0354 treated system. Alternatively, RPS3 changes could simply have a modulating, but not essential, role in this pathway. It must be stressed that further work will be required to definitively show that RPS3 is regulated by IMD0354 and that inhibition of this protein affects MDM2 protein reduction in the context of IKKβ inhibition. Current data shows that RPS3 protein levels appears to be increased over time with IMD0354; however other changes in the tissue culture environment over time have yet to be specifically excluded. Furthermore only one type of siRNA was used in this study which could have non-specific effects therefore further work should aim to confirm this result with a range of RPS3 targeting siRNAs.

This chapter also attempted to identify potential IKKβ substrates by virtue of a phosphoproteome approach. In this approach, cells were treated with IMD0354 or DMSO control and phosphopeptides were extracted using TiO_2 beads. Cells used for this experiments were pre-labelled using stable isotope labelling of cells in culture (SILAC) (*266*). Unfortunately this approach was largely unsuccessful and a number of factors may have contributed to this. One important factor was that trypsin efficiency was very low which leads to a number of problems. This may have occurred as a result of high concentrations of urea in the buffer when trypsinising. Although the concentration of urea within the reaction was reasonably high, this concentration (1.6M) has been shown not to have a detrimental effect on trypsin activity (*294*). Nonetheless as trypsin efficiency was so low, further work should seek to confirm whether this was a confounding factor in the efficiency. If peptides within a sample are not cleaved this leads to much larger peptides than normal. This increases the chance of one or more amino acids not being properly identified in MS2 and thus the data for that peptide being unusable. Additionally, the software used (Protein pilot) is designed to analyse peptides with one SILAC label incorporated. In a normal experiment this is ideal; as arginine and lysine are the labelled amino acids and trypsin cuts after these residues each peptide should contain one label. In certain situations, two labels may be found in one peptide even if trypsin efficiency is good, this is primarily due to the fact that the presence of a proline residue, after either arginine or lysine residues, will prevent cleavage by trypsin and the software is able to cope with this eventuality. However, when trypsin efficiency is poor this can lead to more than two labels per peptide. The large number of mass shifts required to make the peptide fit a normal sequence is then interpreted as making the confidence in the overall sequence lower. This presents a problem in analysing such data.

A further potential problem with this experiment may be that using two different types of peptide clean-up; namely C18 and graphite columns may have increased the loss of peptides from the mixture. A more protracted method with more steps inevitably leads to increased loss of peptides. Graphite columns are

recommended in the purification of phosphopeptides as they are particularly good at binding hydrophilic peptides whereas C18 columns are particularly successful in the purification of hydrophobic peptides (Data from manufacturer: http://www.piercenet.com/browse.cfm?fldID=B5283A74-5056-8A76-4E39-AEC4C18EBE57). Potentially this may mean that sequential use of the two columns increased the loss of peptides as both columns may poorly bind different groups of peptides.

Whilst the data attained from this experiment is of limited use in the exploration of the MDM2-IKKβ axis, the results of this experiment are being used to troubleshoot an improved method of phosphoproteome analysis.

In conclusion, mass spectrometry analysis has shown that a limited number of proteins are altered in the early response to IMD0354 treatment. Screening of a number of these hits led to the identification of RPS3 as a potential regulator of MDM2. Whilst further work will be needed to confirm this, the identification of this protein is of particular interest due to the fact that it has been shown to interact with both the p53 and IKK pathways. This study highlights the usefulness of a proteomics approach to identify proteins or pathways involved in mediating a particular response, in this case response to IKKβ inhibition by IMD0354.

Chapter 6

Conclusions and future perspectives

Chapter 6 Conclusions and future perspectives

The primary aim of this work was to identify and explore dominant regulators of wild-type or mutant p53. Identification of potential factors was conducted by the use of screening methods and included:

1. Screening of compounds to identify those that could activate wtp53
2. Screening of polymers (& hydropolymers) to identify those that could activate wtp53 (or repress activation by Roscovitine)
3. Screening of compounds to identify those that could reduce levels of mtp53

Both screen 1 and screen 3 successfully identified a number of compounds that are potentially able to modify the p53 pathway. Screen 2 aimed to identify polymers which activated p53. Whilst this represents an interesting and novel use of biopolymers, the screen was unfortunately unsuccessful in the identification of such polymers.

The purpose of these various screens was to generate leads of potentially interesting and dominant, compounds or targets, regulating p53. Whilst a huge number of proteins have been shown to affect p53 (*22*) the outcome of perturbation is often difficult to predict. I.e. if a large number of proteins pushes p53 toward degradation and a separate group pushes toward activation this equilibrium is not necessarily going to be substantially altered by perturbing one protein in one of these groups. Screening therefore is a useful way of identifying dominant factors as well as dominant outcomes of target perturbation.

Whilst the screens conducted did lead to the identification of a number of potentially interesting compounds the conclusions that can be made are limited. These screens provided lead generation but further work is needed to confirm whether the hits identified lead to the anticipated outcome. Two compounds which target IKKβ and appeared to be able to reduce mtp53 levels were selected for further examination; the control of the p53 pathway by IKKβ was the subject of further investigation within this project. Nonetheless the identification of the other

hits still represents valuable work. Future studies could explore the control of the p53 pathway by other compounds or hits identified. Particularly the control of the p53 pathway by GSK-3 may be an interesting line of inquiry. Evidence in the literature supports the idea that GSK-3 inhibition could lead to the activation of p53 (*189, 190*). It has therefore been suggested that GSK-3 inhibition may be useful in the treatment of cancers; *in vivo* experiments in mice support this (*189*). However, evidence that GSK-3 is an anti-apoptotic factor is not unequivocal and it has been shown that at least in certain situations GSK-3 can act as a pro-apoptotic factor (*188*). Thus further exploration of this pathway may be beneficial.

Within the confines of this study the influence of IKKβ inhibition on the p53 pathway was selected for further analysis. It was shown that inhibition of IKKβ by IMD0354 led to a reduction of both mtp53 and MDM2. The loss of MDM2 was independent of p53 and was rapidly and acutely affected by IMD0354 and as such the study concentrated on investigating the effects of IMD0354 on MDM2. This study mainly used IMD0354 to inhibit IKKβ, however other IKKβ inhibitors, as well as RNAi, were tested and shown to reduce MDM2 protein levels. The study mainly focussed on using chemical inhibition as the effect of the drug *per se* and it's mechanisms of action are critical to our understanding of pathway manipulation. If IKKβ inhibition is to become a cancer therapy it is likely that such inhibition will be by chemical, not genetic, manipulation. In fact IMD0354 specifically has been proposed as a potential cancer therapy (*229, 230*), although significant work will be required to validate this.

The mechanisms of action of IMD0354 as well as other IKKβ inhibitors are largely explored in the literature in terms of their effects on Nf-κB. In fact IMD0354 is often considered as an Nf-κB specific inhibitor despite the fact that the drug was designed to inhibit IKKβ by binding of the ATP domain (*240*) and IKKβ is known to have a number of other substrate targets (*211*). To our knowledge no study has yet used this drug to investigate the effect on IKKβ dependent, but Nf-κB independent, pathways. This study therefore aimed to take a less bias approach to identification of mediating factors by the use of mass spectrometry. This led to the identification

of RPS3 as a potentially important factor in MDM2 regulation by IMD0354. This is particularly interesting as RPS3 has been previously described as an IKKβ substrate (*281*). Future work should first aim to confirm the effects of RPS3 siRNA on IMD0354 mediated MDM2 reduction using a wider range of siRNAs. Further work should also explore whether IMD0354 is altering the phosphorylation of RPS3 and whether the effects of RPS3 in this pathway involve its ribosomal or non-ribosomal roles.

As a side note, within a separate project it may be interesting to explore the effects of IMD0354 on other described Nf-κB independent IKKβ pathways. Mass spectrometry analysis indicated that pathways involved in Huntington's disease may be up-regulated by the drug. The huntingtin protein has been described as a substrate for IKKβ and IKKβ acts to promote its degradation (*275*). This pathway may provide an interesting starting point in the exploration as to the effects of IKKβ inhibition on non-Nf-κB pathways.

Overall this study demonstrated that IMD0354 appears to be capable of reducing a number of oncogenic proteins including mtp53, MDM2 and Cyclin D1, with MDM2 responding most rapidly. Inhibition of MDM2 may be of great clinical use. There has been much effort within research to identify drugs that inhibit MDM2, although this has largely focussed on inhibiting MDM2 mediated negative regulation of p53. One major issue with this approach is that even in cancers with wtp53 cells, p53 may be supressed by other mechanisms. To date, one drug which inhibits the MDM2 –p53 interaction, Nutlin, has entered clinical trials (*295*). Current available data comes from small scale proof-of-mechanism studies. In one such study a partial response was seen in only 1 of 20 patients (*295*). Clearly larger scale studies are needed to assess clinical outcomes and success in this particular study will have been limited by the short time scale of inhibitor treatment. Nonetheless this suggests that exploration of other drugs targeting MDM2 and the p53 pathway may be beneficial.

A wider approach to MDM2 inhibition would be to reduce MDM2 at the protein level, as is achieved by IMD0354. This would be useful as the oncogenic

capacity of MDM2 is not thought to be limited to only p53 dependent mechanisms. Therefore a reduction in MDM2 at the protein level should reduce all its oncogenic capability. Wang et al. (2001) showed that MDM2 antisense oligonucleotides showed an anticancer effect in treatment of both mice bearing MCF-7 (wtp53) or MDA-MB-468 (mtp53) xenographs (*296*). This suggests that the effect of MDM2 inhibition is not limited to tumours with wtp53. IMD0354 may be of particular use in tumours expressing mtp53 and/or high MDM2 expression as it appears to lead to a reduction in both oncogenic proteins. This study supports the need for future work on the effect of IMD0354 and IKKβ inhibition on MDM2 and mtp53.

Potentially the number of cases where any given MDM2 or p53 directed therapy may be useful will be limited. Cancer is a hugely diverse and heterogeneous disease and whilst pathways such as p53 are often deranged the exact mechanism of this may vary substantially. Therefore much further into the future successful treatment of cancer may require highly personalised therapy. With regards to targeting the p53 pathway this may mean knowing not just the mutational or amplification status of p53 and MDM2, but also the status of a number of other interacting genes. Clearly before this is possible we would need a much greater understanding of why some drugs work in some patients and not others and further increasingly sophisticated and financially viable methods of profiling individual cancers. Nonetheless it is something we could strive towards.

In conclusion, this study identified that IKKβ inhibition by IMD0354 is capable of reducing MDM2, mtp53 and cyclin D in cancer cell lines. Within the p53 pathway MDM2 appears to be the most acutely and rapidly lost. Through the use of mass spectrometry a number of other proteins potentially regulated by IMD0354 treatment were identified and RPS3 was identified as a potential regulator of MDM2 loss. IMD0354 has previously been suggested to be a potential cancer therapy and thus this work suggests that its effects on MDM2 and mtp53 could potentially contribute to an anti-tumorigenic effect and should be an area of future interest. Further work is required to define whether the reduction of MDM2 and/or mtp53

contributes to cell death, to further explore the mechanism of mtp53 and MDM2 reduction and additionally and further in the future, to assess such affects *in vivo*.

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Supplementary Data

Table S1 Tocris Library compounds

Compound Name	Formula	Target	Description
AG 490	C17H14N2O3	EGFR	EGFR-kinase inhibitor. Also JAK2, JAK3 inhibitor
ML 9 hydrochloride	C15H17ClN2O2S.HCl	MLCK	Myosin light chain kinase inhibitor
AG 213	C10H8N2O2S	EGFR	EGFR/PDGFR-kinase inhibitor
Fasudil hydrochloride	C14H17N3O2S.HCl	ROCK	Inhibitor of cyclic nucleotide dependent- and Rho-kinases
GF 109203X	C25H24N4O2	PKC	Protein kinase C inhibitor
Genistein	C15H10O5	EGFR	EGFR kinase inhibitor. Also estrogen and PPARγ ligand
LY 294002 hydrochloride	C19H17NO3.HCl	PI3K	Selective PI 3-kinase inhibitor
U0126	C18H16N6S2	MEK	Potent, selective inhibitor of MEK1 and 2
PD 98059	C16H13NO3	MEK	Specific inhibitor of MEK
Y-27632 dihydrochloride	C14H21N3O.2HCl	ROCK	Selective p160ROCK inhibitor
SB 202190	C20H14N3OF	p38 MAPK	Potent, selective inhibitor of p38 MAPK
Olomoucine	C15H18N6O	cdk	Cyclin-dependent kinase inhibitor
LFM-A13	C11H8Br2N2O2	BTK	Potent, selective BTK inhibitor

ZM 336372	C23H23N3O3	Raf	Potent, selective c-Raf inhibitor
ZM 449829	C13H10O	JAK3	Potent, selective JAK3 inhibitor
ZM 39923 hydrochloride	C23H25NO.HCl	JAK3	Potent, selective JAK3 inhibitor
GW 5074	C15H8Br2INO2	Raf	Potent, selective c-Raf1 kinase inhibitor
PP 1	C16H19N5	Src	Potent, selective Src inhibitor
SB 203580 hydrochloride	C21H16FN3OS.HCl	p38 MAPK	Selective inhibitor of p38 MAPK; water-soluble
(-)-Terreic acid	C7H6O4	BTK	Selective inhibitor of BTK
PP 2	C15H16ClN5	Src	Potent, selective Src inhibitor
SU 4312	C17H16N2O	VEGFR	Potent inhibitor of VEGFR tyrosine kinase
SP 600125	C14H8N2O	JNK	Novel and selective JNK inhibitor
Purvalanol A	C19H25ClN6O	cdk	Cyclin-dependent kinase inhibitor
Purvalanol B	C20H25ClN6O3	cdk	Cyclin-dependent kinase inhibitor
Rottlerin	C30H28O8	PKC	Reported PKCδ inhibitor
SB 431542	C22H16N4O3	TGFbR1	Potent, selective inhibitor of TGF-βRI, ALK4 and ALK7
SB 216763	C19H12Cl2N2O2	GSK-3	Potent, selective GSK-3 inhibitor

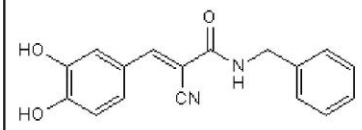
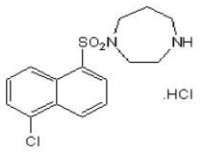
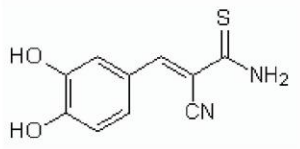
SB 415286	C16H10ClN3O5	GSK-3	Potent, selective GSK-3 inhibitor
Arctigenin	C21H24O6	MEK	Potent MKK1 inhibitor. Also inhibits IκBα phosphorylation
NSC 693868	C9H7N5	cdk	Cdk inhibitor. Also inhibits GSK-3
SB 239063	C20H21FN4O2	p38 MAPK	Potent, selective p38 MAP kinase inhibitor; orally active
SL 327	C16H12F3O3S	MEK	Selective inhibitor of MEK1 and MEK2; brain penetrant
Ro 31-8220 mesylate	C25H23N5O2S.CH3SO3H	Broad Spectrum Inhibitor	Protein kinase inhibitor
Aminopurvalanol A	C19H26ClN7O	cdk	Cyclin-dependent kinase inhibitor
API-2	C13H16N6O4	PKB	Selective inhibitor of Akt/PKB signaling. Antitumor and antiviral
GW 441756	C17H13N3O	TrkA	Potent, selective TrkA inhibitor
GW 583340 dihydrochloride	C28H25ClFN5O3S2.2HCl	EGFR	Potent dual EGFR/ErbB2 inhibitor; orally active
Ro 08-2750	C13H10N4O3	TrkA	Inhibits NGF binding to p75NTR and TrkA
TBB	C6HBr4N3	CK2	Selective cell-permeable Casein Kinase 2 (CK2) inhibitor
1,2,3,4,5,6-Hexabromocyclohexane	C6H6Br6	JAK2	Inhibits JAK2 autophosphorylation
HA 1100 hydrochloride	C14H17N3O3S.HCl	ROCK	Cell-permeable, selective Rho-kinase inhibitor

BIBX 1382 dihydrochloride	C18H19ClFN7.2HCl	EGFR	Highly selective EGFR-kinase inhibitor
CGP 53353	C20H13F2N3O2	PKC	Selective inhibitor of PKCβII
Arcyriaflavin A	C20H11N3O2	cdk	Potent cdk4/cyclin D1 and CaM Kinase II inhibitor. Antiviral agent (anti-HCMV)
ZM 447439	C29H31N5O4	Aurora	Inhibits Aurora kinase B
ER 27319 maleate	C18H20N2O.C4H4O4	Syk	Selective Syk kinase inhibitor
ZM 323881 hydrochloride	C22H18FN3O2.HCl	VEGFR	Potent, selective inhibitor of VEGFR-2
ZM 306416 hydrochloride	C16H13N3O2FCl.HCl	VEGFR	Inhibitor of VEGF receptor tyrosine kinase
IKK 16	C28H29N5OS.HCl	IKK	Selective inhibitor of IKK
Ki 8751	C24H18F3N3O4	VEGFR	Potent, selective VEGFR-2 inhibitor
10-DEBC hydrochloride	C20H25N2OCl.HCl	PKB	Selective Akt/PKB inhibitor
TPCA-1	C12H10FN3O2S	IKK	Potent, selective inhibitor of IKK-2
SB 218078	C24H15N3O3	Chk1	Inhibitor of checkpoint kinase 1 (Chk1)
TCS 359	C18H20N2O4S	FLT3	Potent FLT3 inhibitor
PD 198306	C18H16F3IN2O2	MEK	Selective inhibitor of MEK1/2
Ryuvidine	C15H12N2O2S	cdk	Cyclin-dependent kinase 4 (cdk4) inhibitor

IMD 0354	C15H8ClF6NO2	IKK	Inhibitor of IKK-2
CGK 733	C23H18Cl3FN4O3S	ATR/ATM	Selective inhibitor of ATR and ATM kinases
PHA 665752	C32H34Cl2N4O4S	cMET	Potent and selective MET inhibitor
PD 407824	C20H12N2O3	Chk1	Selective inhibitor of Chk1 and Wee1
LY 364947	C17H12N4	TGFbR1	Selective inhibitor of TGF-βRI
CGP 57380	C11H9FN6	Mnk1	Selective inhibitor of Mnk1
PQ 401	C18H16ClN3O2	IGF-1R	IGF-IR inhibitor
PI 828	C19H18N2O3	PI3K	PI 3-kinase inhibitor, more potent than LY 294002 (Cat.No. 1130)
NU 7026	C17H15NO3	DNA-PK	Selective DNA-dependent protein kinase inhibitor
D 4476	C23H18N4O3	CK1	Selective Casein Kinase 1 (CK1) inhibitor. Also inhibits TGF-β RI
EO 1428	C20H16BrClN2O	p38 MAPK	Selective inhibitor of p38α and p38β2
H 89 dihydrochloride	C20H20BrN3O2S.2HCl	PKA	Protein kinase A inhibitor
FPA 124	C11H9Cl2CuN3O2S	PKB	Akt/PKB inhibitor
GW 843682X	C22H18F3N3O4S	PLK	Selective inhibitor of polo-like kinases, PLK1 and PLK3
Iressa	C22H24ClFN4O3	EGFR	Orally active, selective EGFR inhibitor

SU 5416	C15H14N2O	VEGFR	VEGFR inhibitor. Also inhibits c-kit, Ret, MET and FLT3
1-Naphthyl PP1	C19H19N5	Src	Src family kinase inhibitor; also inhibits c-Abl
Dorsomorphin dihydrochloride	C24H25N5O.2HCl	AMPK	Potent and selective AMPK inhibitor
BIO	C16H10BrN3O6	GSK-3	Potent, selective GSK-3 inhibitor
SD 208	C17H10ClFN6	TGFbR1	Potent ATP-competitive TGF-βRI inhibitor
Compound 401	C16H15N3O2	DNA-PK	Selective DNA-PK and mTOR inhibitor
BI 78D3	C13H9N5O5S2	JNK	Selective, competitive JNK inhibitor
SC 514	C9H8N2OS2	IKK	IKK-2 inhibitor; attenuates NF-κB-induced gene expression

Table S2 Tocris Library compound structures

AG490	ML90 hydrochloride	AG 213
		
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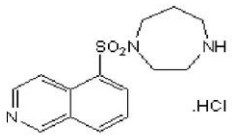	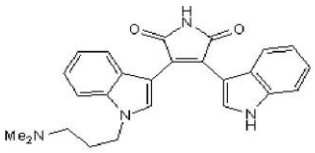	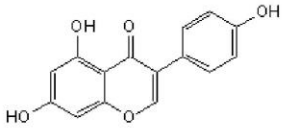
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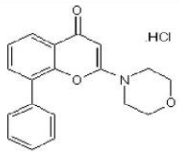	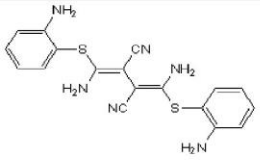	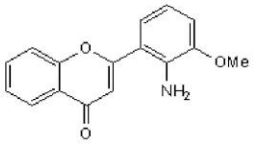
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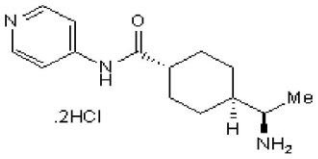	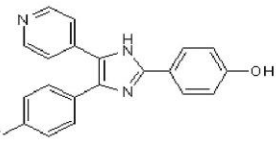	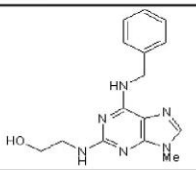
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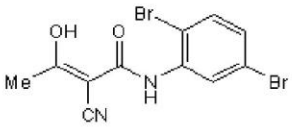	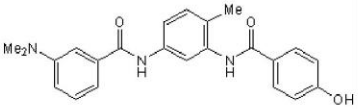	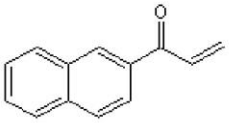
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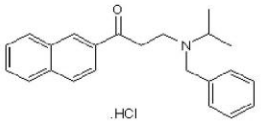	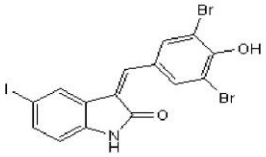	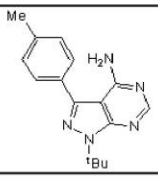
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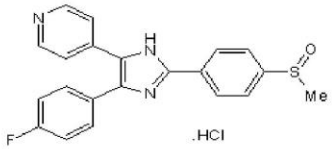	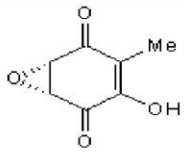	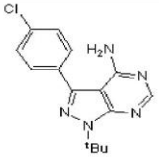
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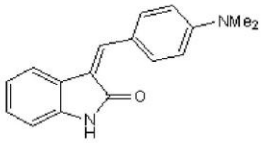	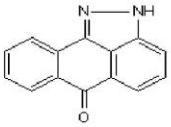	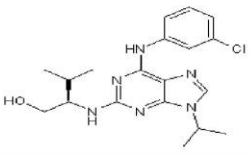

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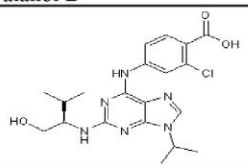	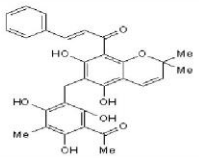	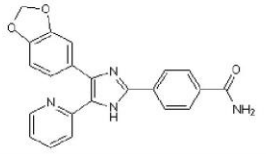
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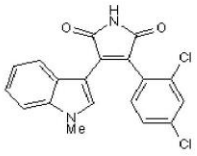	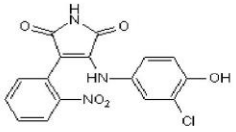	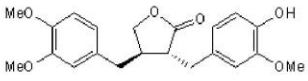
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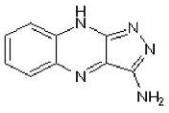	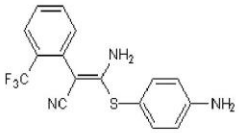	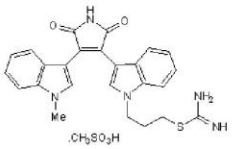
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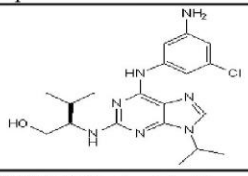	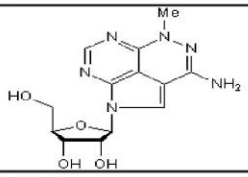	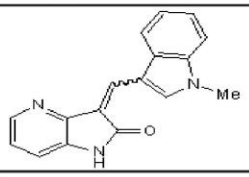
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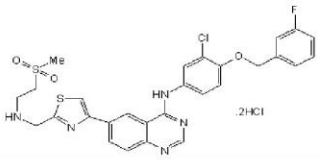	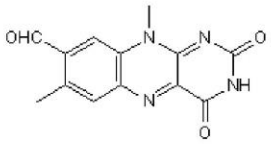	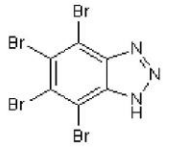
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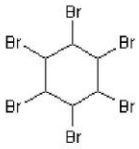	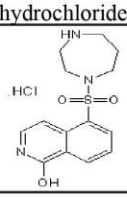	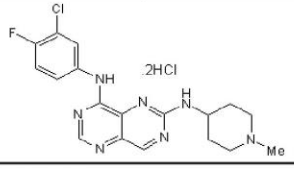
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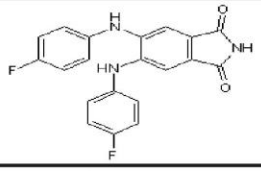	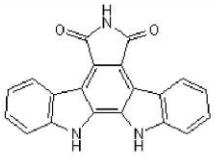	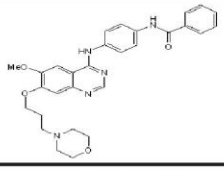
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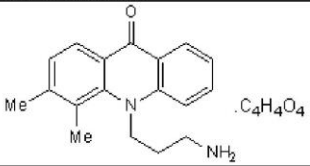	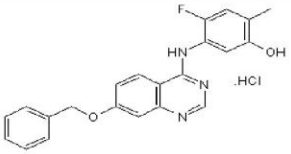	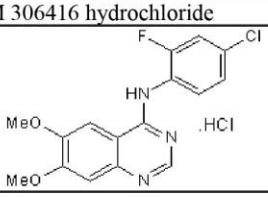

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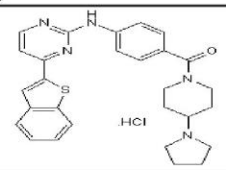	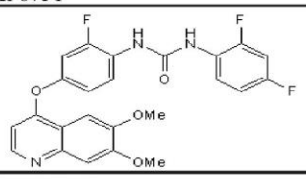	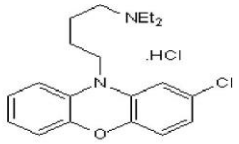
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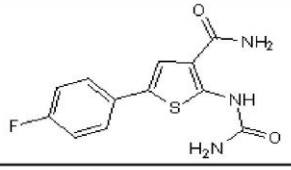	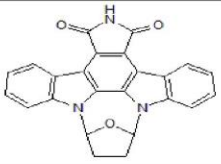	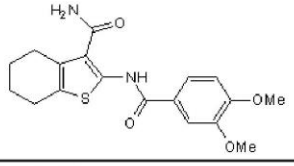
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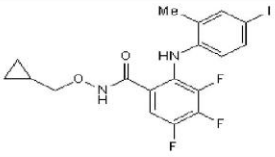	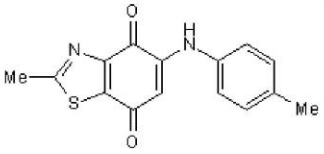	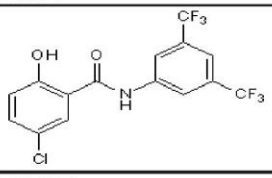
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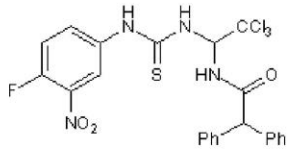	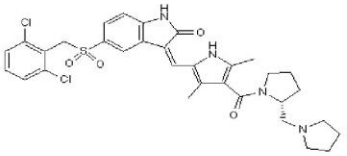	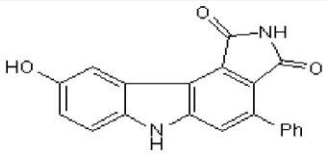
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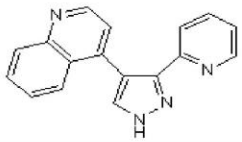	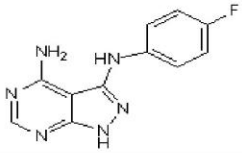	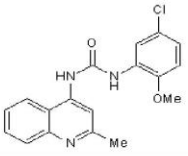
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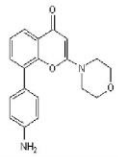	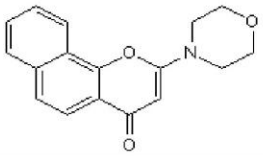	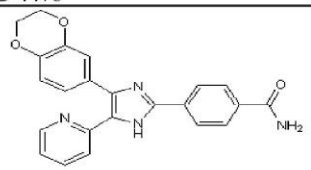
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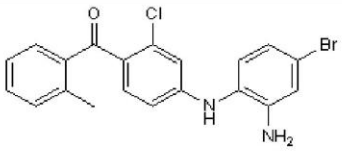	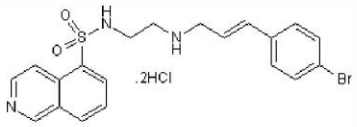	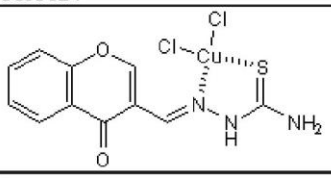
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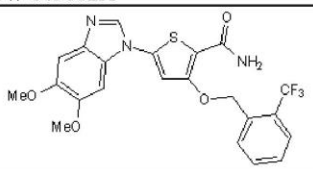
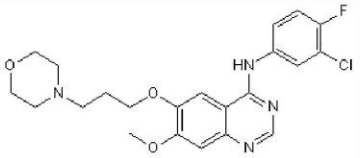
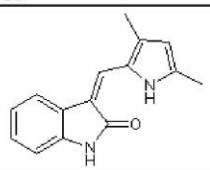
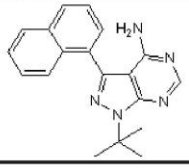
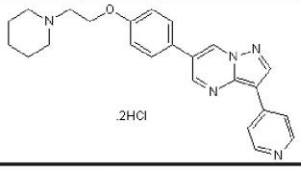
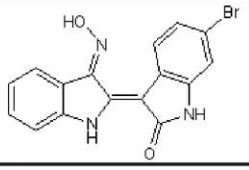
		

1-Naphthyl PP1	Dorsomorphin dihydrochloride	BIO
		
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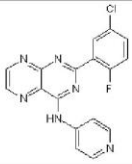
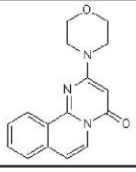
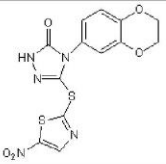
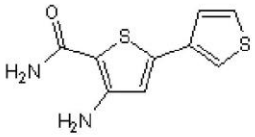
		
SC514		
		

Table S3 Proteins identified as down-regulated (>1.20, Pval <0.05) after IMD0354 treatment using iTRAQ MS/MS

Accession	Symbol	Mass spectrometer	Unused	Peptides 95% Conf.	Cov 95%	30 mins	30mins Pval	60 mins	60mins Pval	120 mins	120mins Pval
O00422	SAP18	ABSciex Qstar Elite	1.45	2	9.15	0.85		1.03		1.03	
		ABSciex 5600	3.69	3	15.03	0.77	0.03	0.94	0.46	0.87	0.15
O00567	NOP56	ABSciex Qstar Elite	14.84	7	17.17	0.76	0.00	0.98	0.70	0.95	0.39
		ABSciex 5600	6.37	3	7.24	0.78	0.11	1.05	0.61	0.96	0.68
O14979	HNRDL	ABSciex Qstar Elite	14.57	8	19.76	0.73	0.06	0.95	0.61	0.88	0.34
		ABSciex 5600	16.41	8	19.05	0.69	0.01	0.97	0.80	0.91	0.43
O15020	SPTN2	ABSciex Qstar Elite	35.51	19	11.72	0.90	0.06	0.97	0.51	0.91	0.02
		ABSciex 5600	15.23	10	5.40	0.92	0.20	0.96	0.50	0.81	0.00
O60716	CTNND1	ABSciex Qstar Elite	35.52	17	26.65	1.00	0.93	0.88	0.01	0.82	0.00
		ABSciex 5600	40.2	20	28.10	0.98	0.51	0.87	0.00	0.81	0.00
O75367	H2AY	ABSciex Qstar Elite	33.3	19	40.05	0.75	0.00	1.05	0.35	0.96	0.54
		ABSciex 5600	21.86	15	34.68	0.63	0.00	1.08	0.23	0.88	0.10
O75533	SF3B1	ABSciex Qstar Elite	23.86	12	13.27	0.88	0.01	1.06	0.14	1.02	0.62
		ABSciex 5600	16.31	11	8.67	0.83	0.01	0.96	0.46	0.95	0.39
O75643	snRNP 200	ABSciex Qstar Elite	14.64	7	4.26	0.84	0.02	0.92	0.22	0.89	0.10
		ABSciex 5600	8.36	5	2.95	0.81	0.04	1.00	0.97	0.84	0.12
O75976	CBPD	ABSciex Qstar Elite	14.17	7	6.16	0.72	0.00	0.76	0.10	0.69	0.04
		ABSciex 5600	10.38	5	5.87	0.93	0.32	1.08	0.55	0.82	0.06
O95831	AIFM1	ABSciex Qstar Elite	13.71	7	14.52	0.88	0.08	0.97	0.65	1.00	0.95

		ABSciex 5600	12.72	6	12.40	0.75	0.01	0.94	0.38	0.94	0.34
P06576	ATPB	ABSciex Qstar Elite	50.17	56	57.66	0.74	0.00	0.97	0.47	0.92	0.04
		ABSciex 5600	40.79	36	50.85	0.62	0.00	1.00	0.96	0.89	0.02
P07305	H10	ABSciex Qstar Elite	7.39	5	22.68	0.82	0.15	0.98	0.86	0.90	0.35
		ABSciex 5600	7.33	4	20.62	0.71	0.01	0.92	0.32	0.86	0.09
P07910	hnRNPC	ABSciex Qstar Elite	21.46	13	35.95	0.70	0.00	1.00	0.98	0.94	0.45
		ABSciex 5600	26.15	17	42.81	0.46	0.00	1.10	0.27	0.93	0.43
P08621	RU17	ABSciex Qstar Elite	7.92	4	10.76	0.85	0.25	1.01	0.94	1.03	0.82
		ABSciex 5600	11.62	6	13.73	0.76	0.01	0.99	0.82	0.98	0.75
P09382	LEG1	ABSciex Qstar Elite	15.95	11	70.37	0.86	0.07	1.05	0.54	0.96	0.53
		ABSciex 5600	14.33	10	64.44	0.69	0.00	1.00	0.97	0.93	0.33
P09669	COX6C	ABSciex Qstar Elite	1.92	1	10.67	0.72	0.24	1.08	0.70	1.04	0.80
		ABSciex 5600	4.81	3	33.33	0.73	0.04	1.06	0.75	0.96	0.76
P12270	TPR	ABSciex Qstar Elite	40.77	20	12.57	0.82	0.00	0.89	0.00	0.91	0.03
		ABSciex 5600	33.05	18	10.11	0.88	0.02	1.01	0.78	0.96	0.43
P12830	CADH1	ABSciex Qstar Elite	31.91	23	28.57	0.91	0.09	0.90	0.26	0.81	0.00
		ABSciex 5600	20.64	9	13.38	0.90	0.07	0.88	0.03	0.82	0.00
P14678	RSMB	ABSciex Qstar Elite	7.76	5	15.83	0.78	0.12	0.93	0.45	0.88	0.21
		ABSciex 5600	5.39	4	12.08	0.77	0.04	0.91	0.31	1.03	0.92
P14866	HNRPL	ABSciex Qstar Elite	24.75	15	29.88	0.81	0.00	1.01	0.81	0.94	0.23
		ABSciex 5600	23.42	13	24.62	0.58	0.00	1.01	0.91	0.88	0.21
P14923	PLAK	ABSciex Qstar Elite	27.25	17	28.72	0.95	0.59	0.87	0.02	0.81	0.03

		ABSciex 5600	17.5	10	15.30	0.89	0.11	0.88	0.07	0.80	0.02
P16401	H15	ABSciex Qstar Elite	21.25	19	39.82	0.77	0.03	0.88	0.15	0.86	0.13
		ABSciex 5600	11.27	9	32.30	0.78	0.38	0.88	0.63	0.84	0.54
P17480	UBF1	ABSciex Qstar Elite	12.65	7	10.08	0.86	0.06	0.87	0.08	0.75	0.01
		ABSciex 5600	4.67	2	3.66	0.76	0.04	0.82	0.09	0.75	0.05
P18583	SON	ABSciex Qstar Elite	19.65	10	5.40	0.77	0.00	0.98	0.62	0.95	0.32
		ABSciex 5600	14.45	8	4.20	0.79	0.00	1.02	0.75	1.03	0.52
P18859	ATP5J	ABSciex Qstar Elite	8.46	5	40.74	0.74	0.02	1.06	0.50	1.02	0.77
		ABSciex 5600	6.53	4	39.81	0.72	0.15	1.14	0.26	0.98	0.87
P19105	ML12A	ABSciex Qstar Elite	16.79	9	47.37	0.92	0.63	0.86	0.42	0.76	0.27
		ABSciex 5600	15.83	8	55.56	1.01	0.90	0.94	0.38	0.83	0.02
P22087	FBRL	ABSciex Qstar Elite	7.29	5	16.82	0.78	0.04	0.93	0.42	0.88	0.17
		ABSciex 5600	5.1	3	9.97	0.62	0.04	0.82	0.13	0.78	0.10
P22626	ROA2	ABSciex Qstar Elite	38.37	28	56.37	0.69	0.00	0.93	0.16	0.90	0.04
		ABSciex 5600	29.39	45	54.96	0.53	0.00	0.98	0.81	0.83	0.04
P23246	SFPQ	ABSciex Qstar Elite	30.17	18	20.93	0.82	0.00	1.14	0.01	1.09	0.06
		ABSciex 5600	27.9	15	22.91	0.87	0.01	1.16	0.00	1.12	0.02
P25705	ATPA	ABSciex Qstar Elite	43.61	31	43.76	0.73	0.00	0.97	0.44	0.93	0.07
		ABSciex 5600	38.26	27	30.74	0.62	0.00	1.05	0.64	0.99	0.94
P26599	PTBP1	ABSciex Qstar Elite	14.25	8	17.14	0.89	0.20	1.04	0.62	0.96	0.59
		ABSciex 5600	15.16	7	16.38	0.81	0.04	1.03	0.70	0.95	0.51
P27824	CALX	ABSciex Qstar Elite	35.86	20	31.93	0.82	0.00	1.04	0.25	1.03	0.62

		ABSciex 5600	26.99	14	26.18	0.76	0.00	1.03	0.64	1.01	0.87
P31942	HNRH3	ABSciex Qstar Elite	12.07	8	23.70	0.72	0.01	0.97	0.71	0.94	0.39
		ABSciex 5600	7.77	6	24.57	0.67	0.05	0.94	0.49	0.86	0.15
P36957	ODO2	ABSciex Qstar Elite	18.28	12	22.96	0.81	0.00	0.90	0.08	0.90	0.09
		ABSciex 5600	14.98	9	18.76	0.81	0.02	0.90	0.11	0.89	0.26
P38159	hnRNPG	ABSciex Qstar Elite	19.61	9	28.13	0.63	0.00	0.91	0.20	0.82	0.01
		ABSciex 5600	29.08	12	28.39	0.58	0.00	1.01	0.70	0.87	0.00
P43243	MATR3	ABSciex Qstar Elite	32.63	21	23.49	0.83	0.00	0.93	0.08	0.86	0.00
		ABSciex 5600	36.91	22	24.32	0.78	0.00	0.96	0.29	0.86	0.01
P49756	RBM25	ABSciex Qstar Elite	19.26	11	16.61	0.77	0.00	0.98	0.68	0.96	0.44
		ABSciex 5600	17.94	12	14.35	0.69	0.00	0.93	0.25	0.88	0.20
P51991	ROA3	ABSciex Qstar Elite	10.03	8	21.69	0.75	0.04	0.96	0.63	0.93	0.47
		ABSciex 5600	7.33	9	19.58	0.61	0.19	0.91	0.56	0.78	0.27
P58876	H2B1D	ABSciex Qstar Elite	19.39	28	57.14	0.76	0.02	1.06	0.45	0.99	0.95
		ABSciex 5600	0	20	50.79						
P60660	MYL6	ABSciex Qstar Elite	16.2	13	52.32	0.89	0.08	0.91	0.13	0.83	0.01
		ABSciex 5600	15.36	10	56.95	0.89	0.10	0.94	0.41	0.83	0.02
P62979	RS27A	ABSciex Qstar Elite	15.07	15	37.82	0.90	0.11	0.95	0.38	0.83	0.02
		ABSciex 5600	15.69	14	41.67	0.92	0.27	0.99	0.88	0.77	0.03
P82979	SARNP	ABSciex Qstar Elite	10.66	6	38.57	0.81	0.03	0.92	0.30	0.91	0.20
		ABSciex 5600	11.08	8	31.43	0.82	0.05	0.91	0.19	0.90	0.15
P84103	SFRS3	ABSciex Qstar Elite	17.51	10	43.29	0.81	0.02	0.96	0.55	0.92	0.34

		ABSciex 5600	17.09	8	43.29	0.70	0.07	1.02	0.87	0.88	0.48
P98175	RBM10	ABSciex Qstar Elite	12.53	6	7.20	0.81	0.01	0.86	0.04	0.85	0.03
		ABSciex 5600	6.96	4	4.41	0.86	0.09	0.82	0.05	0.79	0.16
Q01826	SATB1	ABSciex Qstar Elite	12.54	8	13.24	0.74	0.01	0.76	0.03	0.71	0.01
		ABSciex 5600	13.87	8	14.55	0.87	0.20	0.92	0.48	0.87	0.15
Q04837	SSBP	ABSciex Qstar Elite	18.15	11	57.43	0.80	0.01	0.87	0.05	0.79	0.02
		ABSciex 5600	6.02	5	33.78	0.72	0.24	0.87	0.46	0.75	0.27
Q07021	C1QBP	ABSciex Qstar Elite	12.04	6	31.21	0.71	0.01	1.00	0.99	1.04	0.70
		ABSciex 5600	5.26	3	15.60	0.67	0.16	1.02	0.95	1.13	0.72
Q07065	CKAP4	ABSciex Qstar Elite	30.98	15	32.89	0.82	0.00	1.03	0.50	1.06	0.19
		ABSciex 5600	23.85	11	23.75	0.78	0.00	1.05	0.32	1.03	0.58
Q07955	SFRS1	ABSciex Qstar Elite	18.46	12	42.34	0.80	0.00	0.89	0.10	0.87	0.03
		ABSciex 5600	20.7	13	46.77	0.70	0.00	0.86	0.06	0.81	0.00
Q08379	GOLGA2	ABSciex Qstar Elite	13.45	8	10.08	0.90	0.15	0.78	0.01	0.80	0.03
		ABSciex 5600	18.69	11	12.48	0.84	0.03	0.81	0.02	0.82	0.06
Q12874	SF3A3	ABSciex Qstar Elite	12.77	8	18.96	0.82	0.04	0.88	0.08	0.86	0.06
		ABSciex 5600	6	3	10.18	0.82	0.19	0.92	0.57	0.87	0.40
Q12888	TP53B	ABSciex Qstar Elite	27.15	15	14.20	0.82	0.01	0.90	0.07	0.93	0.19
		ABSciex 5600	21.13	14	10.45	0.83	0.04	0.90	0.09	0.95	0.61
Q12906	ILF3	ABSciex Qstar Elite	24.46	14	19.24	0.76	0.00	0.96	0.34	0.93	0.12
		ABSciex 5600	28.68	14	19.24	0.75	0.00	0.91	0.04	0.86	0.00
Q13185	CBX3	ABSciex Qstar Elite	6.33	3	22.40	0.87	0.40	1.04	0.76	0.97	0.86

		ABSciex 5600	8.24	5	25.14	0.62	0.00	0.89	0.19	0.82	0.04
Q13242	SFRS9	ABSciex Qstar Elite	17.45	10	40.72	0.72	0.00	0.93	0.21	0.83	0.02
		ABSciex 5600	11.9	6	26.24	0.69	0.00	0.88	0.10	0.77	0.01
Q13435	SF3B2	ABSciex Qstar Elite	38.94	24	30.28	0.77	0.00	0.91	0.08	0.89	0.03
		ABSciex 5600	41.12	21	23.58	0.77	0.00	0.88	0.00	0.84	0.00
Q13573	SNW1	ABSciex Qstar Elite	12.03	6	13.62	0.77	0.02	0.92	0.28	0.92	0.28
		ABSciex 5600	11.37	7	16.23	0.76	0.00	0.90	0.08	0.91	0.21
Q13813	SPTA2	ABSciex Qstar Elite	108.94	53	26.98	0.83	0.00	0.93	0.00	0.86	0.00
		ABSciex 5600	96.23	55	26.01	0.81	0.00	0.92	0.02	0.84	0.00
Q14126	DSG2	ABSciex Qstar Elite	14.22	7	11.00	0.84	0.03	0.77	0.01	0.76	0.00
		ABSciex 5600	6.1	4	3.94	0.92	0.66	0.91	0.68	0.85	0.37
Q14151	SAFB2	ABSciex Qstar Elite	5.28	6	8.92	0.72	0.06	1.16	0.23	0.96	0.72
		ABSciex 5600	2.54	7	9.65	0.67	0.01	1.04	0.72	0.77	0.14
Q14257	RCN2	ABSciex Qstar Elite	18.09	10	30.60	0.97	0.65	0.93	0.33	0.99	0.83
		ABSciex 5600	9.66	6	19.56	0.93	0.30	0.82	0.04	0.91	0.22
Q14980	NUMA1	ABSciex Qstar Elite	81.97	41	26.19	0.83	0.00	1.01	0.75	0.98	0.54
		ABSciex 5600	80.58	43	26.81	0.79	0.00	1.01	0.78	0.97	0.42
Q15424	SAFB	ABSciex Qstar Elite	12.47	7	10.38	0.71	0.02	1.01	0.91	0.92	0.31
		ABSciex 5600	19.57	9	15.41	0.64	0.04	0.92	0.55	0.78	0.13
Q15459	SF3A1	ABSciex Qstar Elite	24.81	12	21.06	0.76	0.00	0.86	0.02	0.86	0.03
		ABSciex 5600	16.45	9	13.49	0.80	0.00	0.93	0.30	0.93	0.22
Q16629	SFRS7	ABSciex Qstar Elite	12.97	11	27.31	0.74	0.00	0.95	0.28	0.89	0.11

		ABSciex 5600	12.78	8	20.59	0.65	0.00	0.88	0.11	0.79	0.05
Q1KMD3	hnRNPUL2	ABSciex Qstar Elite	21.62	11	20.21	0.80	0.00	0.88	0.01	0.87	0.01
		ABSciex 5600	26.04	14	20.75	0.80	0.02	0.94	0.40	0.89	0.12
Q6PJT7	ZC3H14	ABSciex Qstar Elite	8.18	4	8.15	0.81	0.03	0.94	0.43	0.92	0.30
		ABSciex 5600	11.79	6	10.19	0.79	0.01	0.90	0.14	0.94	0.38
Q86UE4	LYRIC	ABSciex Qstar Elite	17.17	9	19.93	0.84	0.01	1.02	0.68	1.01	0.84
		ABSciex 5600	17.97	9	20.10	0.80	0.00	0.99	0.88	1.00	0.98
Q86UP2	KTN1	ABSciex Qstar Elite	47.61	23	19.60	0.80	0.00	1.00	0.89	0.97	0.49
		ABSciex 5600	54.91	31	24.02	0.75	0.00	1.00	0.96	0.94	0.20
Q86V81	THOC4	ABSciex Qstar Elite	12.37	7	37.74	0.77	0.04	0.95	0.56	0.93	0.42
		ABSciex 5600	12.65	10	37.74	0.79	0.06	1.06	0.56	0.99	0.88
Q86VM9	ZCH18	ABSciex Qstar Elite	5.26	3	5.46	0.90	0.59	0.96	0.68	0.96	0.67
		ABSciex 5600	7.47	5	4.62	0.58	0.02	1.12	0.27	1.03	0.74
Q8IWX8	CHERP	ABSciex Qstar Elite	9.49	5	6.55	0.82	0.04	0.90	0.12	0.84	0.02
		ABSciex 5600	2.34	2	2.29	0.92	0.72	1.05	0.70	1.08	0.79
Q8IX12	CCAR1	ABSciex Qstar Elite	14.35	8	8.78	0.77	0.00	0.94	0.29	0.89	0.06
		ABSciex 5600	6.62	6	4.78	0.71	0.02	0.93	0.33	0.88	0.26
Q8IYB3	SRRM1	ABSciex Qstar Elite	6.21	6	7.41	0.78	0.09	1.01	0.92	1.03	0.77
		ABSciex 5600	11.07	5	7.96	0.78	0.01	1.00	0.97	1.03	0.76
Q8TAQ2	SMARCC2	ABSciex Qstar Elite	14	8	9.31	0.83	0.03	0.88	0.09	0.85	0.05
		ABSciex 5600	19.09	10	10.96	0.81	0.04	0.94	0.57	0.94	0.35
Q92896	GSLG1	ABSciex Qstar Elite	12.39	7	6.53	0.92	0.37	0.96	0.53	0.78	0.03

		ABSciex 5600	9.95	6	4.24	0.93	0.43	0.96	0.44	0.79	0.02
Q96AQ6	PBXIP1	ABSciex Qstar Elite	11.72	6	10.94	0.72	0.02	0.82	0.13	0.75	0.03
		ABSciex 5600	14.21	8	14.23	0.95	0.71	1.00	0.99	1.04	0.78
Q96EP5	DAZP1	ABSciex Qstar Elite	8.67	4	14.00	0.79	0.03	0.84	0.07	0.87	0.24
		ABSciex 5600	2.95	1	3.93	0.79	0.05	0.86	0.33	0.85	0.10
Q99459	CDC5L	ABSciex Qstar Elite	16.39	9	17.08	0.88	0.13	0.99	0.84	0.97	0.56
		ABSciex 5600	21.47	11	18.20	0.79	0.00	0.93	0.20	0.89	0.03
Q9BUP0	EFHD1	ABSciex Qstar Elite	13.36	7	41.00	1.17	0.39	0.81	0.03	0.71	0.01
		ABSciex 5600	4.29	4	12.97	1.14	0.30	0.77	0.12	0.62	0.13
Q9BZQ8	NIBAN	ABSciex Qstar Elite	0	0							
		ABSciex 5600	2.03	1	2.26	0.65	0.04	0.86	0.59	0.89	0.73
Q9H307	PININ	ABSciex Qstar Elite	19.22	12	20.78	0.79	0.01	0.98	0.79	0.98	0.82
		ABSciex 5600	18.41	10	13.25	0.79	0.00	1.01	0.76	0.95	0.41
Q9NX14	NDUBB	ABSciex Qstar Elite	7.35	4	37.91	0.82	0.07	0.93	0.39	0.76	0.04
		ABSciex 5600	4.08	3	18.30	0.74	0.19	0.84	0.41	0.64	0.20
Q9UKM9	RALY	ABSciex Qstar Elite	11.45	6	17.32	0.71	0.00	0.97	0.72	0.88	0.06
		ABSciex 5600	12.69	6	17.97	0.59	0.00	0.99	0.91	0.86	0.04
Q9UKV3	ACIN	ABSciex Qstar Elite	16.99	11	9.77	0.64	0.00	0.88	0.07	0.87	0.04
		ABSciex 5600	21	13	8.95	0.64	0.00	0.92	0.14	0.87	0.11
Q9UMS4	PRP19	ABSciex Qstar Elite	14.65	9	25.79	0.80	0.03	0.99	0.92	0.94	0.45
		ABSciex 5600	8.98	7	18.65	0.77	0.05	1.00	1.00	0.93	0.47
Q9UPT8	ZC3H4	ABSciex Qstar Elite	4.09	2	2.69	0.87	0.41	1.02	0.86	0.96	0.75

		ABSciex 5600	3.86	2	1.84	0.78	0.05	0.86	0.19	1.02	0.79
Q9UQ35	SRRM2	ABSciex Qstar Elite	51.2	27	15.66	0.83	0.00	0.98	0.64	0.98	0.56
		ABSciex 5600	54.02	33	15.08	0.75	0.00	0.98	0.69	1.00	0.91
Q9Y2X3	NOP58	ABSciex Qstar Elite	13.36	6	15.50	0.75	0.03	0.97	0.62	0.91	0.17
		ABSciex 5600	6.32	5	12.10	0.76	0.20	0.90	0.30	0.96	0.83
Q9Y3Y2	FOP	ABSciex Qstar Elite	6.09	3	16.94	0.68	0.03	0.96	0.55	0.90	0.19
		ABSciex 5600	5	3	16.94	0.78	0.02	0.95	0.48	0.97	0.73

Table S4 Proteins identified as Up-regulated (<0.83, Pval <0.05) after IMD0354 treatment using iTRAQ MS/MS

Accession	Symbol	Mass spectrometer	Unused	Peptides 95% Conf.	Cov 95%	30mins	30mins Pval	60mins	60mins Pval	120mins	120mins Pval
P00338	LDHA	ABSciex Qstar Elite	28.12	15	38.25	1.14	0.02	1.12	0.04	1.18	0.02
		ABSciex 5600	29.63	19	35.84	1.28	0.00	1.25	0.01	1.28	0.01
P00558	PGK1	ABSciex Qstar Elite	46.1	29	58.99	1.11	0.01	1.07	0.05	1.05	0.18
		ABSciex 5600	45.85	26	49.64	1.21	0.00	1.12	0.01	1.13	0.02
P04406	G3P	ABSciex Qstar Elite	41.23	45	65.07	1.02	0.81	0.96	0.70	0.99	0.91
		ABSciex 5600	46.15	35	62.69	1.21	0.03	1.16	0.13	1.16	0.14
P05388	RLA0	ABSciex Qstar Elite	26.85	15	43.53	1.09	0.09	1.02	0.68	1.02	0.60
		ABSciex 5600	16.43	9	36.59	1.28	0.00	1.03	0.53	1.11	0.08
P06733	ENOA	ABSciex Qstar Elite	51.04	37	55.76	1.09	0.10	1.04	0.34	1.06	0.15
		ABSciex 5600	46.46	30	50.69	1.43	0.00	1.30	0.00	1.27	0.00
P07737	PROF1	ABSciex Qstar Elite	19.81	12	68.57	1.11	0.08	1.09	0.15	1.09	0.18
		ABSciex 5600	18	11	68.57	1.26	0.01	1.15	0.13	1.21	0.09
P07741	APT	ABSciex Qstar Elite	11.25	10	36.67	1.16	0.12	1.14	0.16	1.17	0.11
		ABSciex 5600	9.46	6	28.89	1.29	0.02	1.25	0.03	1.28	0.02
P07900	HS90A	ABSciex Qstar Elite	107.11	91	58.33	1.04	0.41	1.01	0.85	1.02	0.70
		ABSciex 5600	85.01	78	49.18	1.23	0.01	1.15	0.08	1.14	0.15
P08133	ANXA6	ABSciex Qstar Elite	64.25	38	49.03	1.18	0.00	1.12	0.01	1.15	0.00

		ABSciex 5600	61.27	32	48.29	1.23	0.00	1.13	0.01	1.16	0.00
P08238	HS90B	ABSciex Qstar Elite	47.54	82	56.91	1.05	0.30	1.03	0.56	1.05	0.43
		ABSciex 5600	44.69	69	48.62	1.26	0.02	1.12	0.21	1.11	0.21
P08758	ANXA5	ABSciex Qstar Elite	29.18	22	52.19	1.22	0.00	1.08	0.20	1.08	0.19
		ABSciex 5600	19.31	11	34.69	1.16	0.08	1.07	0.50	1.04	0.53
P13489	RINI	ABSciex Qstar Elite	14.25	8	17.79	1.17	0.02	1.11	0.10	1.14	0.10
		ABSciex 5600	13.15	9	13.45	1.23	0.02	1.12	0.28	1.13	0.18
P13639	EF2	ABSciex Qstar Elite	83.83	56	44.87	1.10	0.00	1.07	0.03	1.08	0.02
		ABSciex 5600	66.28	42	35.43	1.24	0.00	1.21	0.01	1.21	0.00
P14618	KPYM	ABSciex Qstar Elite	62.19	45	62.15	1.12	0.00	1.07	0.11	1.07	0.05
		ABSciex 5600	49.58	31	45.39	1.28	0.00	1.20	0.01	1.10	0.14
P21266	GSTM3	ABSciex Qstar Elite	15.91	11	42.67	1.35	0.00	1.30	0.01	1.23	0.03
		ABSciex 5600	3.58	3	12.89	1.38	0.12	1.35	0.05	1.28	0.07
P22234	PUR6	ABSciex Qstar Elite	23.34	11	26.82	1.14	0.11	1.11	0.09	1.13	0.05
		ABSciex 5600	12.91	9	12.94	1.14	0.15	1.16	0.05	1.26	0.03
P23396	RS3	ABSciex Qstar Elite	20.63	14	44.86	1.21	0.01	1.04	0.44	1.11	0.21
		ABSciex 5600	10.31	5	24.28	1.29	0.03	1.02	0.81	1.08	0.51
P25789	PSA4	ABSciex Qstar Elite	14.22	8	26.82	1.08	0.22	1.07	0.22	1.10	0.18
		ABSciex 5600	12.35	6	21.07	1.16	0.27	1.15	0.07	1.26	0.05
P26639	SYTC	ABSciex Qstar Elite	20.14	10	16.18	1.10	0.11	1.12	0.03	1.11	0.05

		ABSciex 5600	9.69	5	9.27	1.23	0.02	1.06	0.38	1.09	0.30
P29373	RABP2	ABSciex Qstar Elite	12.67	7	55.80	1.13	0.07	1.13	0.06	1.11	0.11
		ABSciex 5600	6.5	4	33.33	1.24	0.02	1.20	0.04	1.22	0.03
P29401	TKT	ABSciex Qstar Elite	34.81	29	32.74	1.00	0.95	1.02	0.69	1.06	0.29
		ABSciex 5600	35.34	20	28.25	1.08	0.07	1.05	0.19	1.22	0.00
P30041	PRDX6	ABSciex Qstar Elite	32.55	24	62.95	1.14	0.01	1.09	0.05	1.09	0.11
		ABSciex 5600	24.2	15	49.55	1.13	0.20	1.26	0.03	1.07	0.36
P30043	BLVRB	ABSciex Qstar Elite	9.4	7	31.55	1.21	0.05	1.14	0.13	1.11	0.19
		ABSciex 5600	7.77	6	28.16	1.16	0.11	1.09	0.30	1.05	0.50
P30044	PRDX5	ABSciex Qstar Elite	16.03	8	44.39	1.09	0.19	1.11	0.18	1.12	0.14
		ABSciex 5600	12	7	36.45	1.23	0.02	1.19	0.07	1.22	0.02
P32119	PRDX2	ABSciex Qstar Elite	15.12	11	37.88	1.04	0.49	1.08	0.19	1.09	0.17
		ABSciex 5600	16	11	35.86	1.20	0.02	1.16	0.05	1.20	0.02
P36405	ARL3	ABSciex Qstar Elite	14.65	8	53.30	1.20	0.04	1.20	0.04	1.14	0.13
		ABSciex 5600	11.98	6	40.11	1.39	0.06	1.30	0.04	1.15	0.14
P37837	TALDO	ABSciex Qstar Elite	20.53	14	37.09	1.09	0.07	1.05	0.27	1.08	0.09
		ABSciex 5600	23.54	14	32.34	1.29	0.01	1.23	0.02	1.32	0.00
P39019	RS19	ABSciex Qstar Elite	15.01	9	42.76	1.09	0.16	1.11	0.10	1.14	0.06
		ABSciex 5600	15.99	8	48.28	1.39	0.01	1.32	0.02	1.46	0.01
P39023	RL3	ABSciex Qstar Elite	13.91	7	21.09	1.04	0.46	1.08	0.15	1.19	0.01

		ABSciex 5600	12.62	8	18.86	1.11	0.18	1.19	0.04	1.36	0.01
P46781	RS9	ABSciex Qstar Elite	17.44	9	32.47	1.26	0.02	1.20	0.01	1.25	0.02
		ABSciex 5600	10.31	6	20.62	1.19	0.18	1.14	0.40	1.23	0.12
P46782	RS5	ABSciex Qstar Elite	17.75	15	47.55	1.08	0.21	0.98	0.70	1.06	0.32
		ABSciex 5600	17.48	10	39.71	1.22	0.01	1.20	0.02	1.11	0.12
P46783	RS10	ABSciex Qstar Elite	8.08	5	32.73	1.18	0.23	1.14	0.25	1.14	0.13
		ABSciex 5600	5.33	3	19.39	1.22	0.04	1.17	0.07	1.20	0.04
P47756	CAPZB	ABSciex Qstar Elite	10.05	6	25.27	1.09	0.26	1.02	0.83	1.05	0.57
		ABSciex 5600	7.46	4	15.16	1.24	0.04	1.06	0.47	1.07	0.41
P49189	AL9A1	ABSciex Qstar Elite	16.96	12	26.32	1.05	0.53	1.07	0.34	1.06	0.40
		ABSciex 5600	19.88	10	21.26	1.06	0.48	1.11	0.16	1.21	0.04
P49207	RL34	ABSciex Qstar Elite	6.81	4	28.21	0.91	0.20	1.15	0.20	1.32	0.07
		ABSciex 5600	9.28	5	35.90	1.07	0.41	1.24	0.05	1.43	0.01
P49327	FASN	ABSciex Qstar Elite	140.59	80	33.05	1.11	0.00	1.06	0.01	1.05	0.02
		ABSciex 5600	90.97	61	23.74	1.25	0.00	1.14	0.00	1.15	0.00
P49720	PSB3	ABSciex Qstar Elite	15.43	8	45.85	1.27	0.01	1.13	0.15	1.23	0.06
		ABSciex 5600	4.7	3	21.46	1.30	0.09	1.05	0.68	1.17	0.21
P52209	6PGD	ABSciex Qstar Elite	12.46	9	22.36	1.10	0.15	1.10	0.17	1.06	0.38
		ABSciex 5600	14.32	8	21.74	1.21	0.01	1.09	0.18	1.10	0.16
P54578	UBP14	ABSciex Qstar Elite	9.34	5	10.73	1.07	0.35	1.05	0.50	1.04	0.67

		ABSciex 5600	4.8	3	6.88	1.28	0.04	1.16	0.25	1.17	0.12
P55060	XPO2	ABSciex Qstar Elite	32.02	18	17.51	1.21	0.00	1.23	0.00	1.16	0.00
		ABSciex 5600	22.06	11	9.99	1.32	0.00	1.28	0.00	1.24	0.01
P62195	PRS8	ABSciex Qstar Elite	13.38	10	28.57	1.26	0.02	1.19	0.05	1.23	0.08
		ABSciex 5600	8.74	6	16.75	1.23	0.04	1.17	0.09	1.11	0.22
P62249	RS16	ABSciex Qstar Elite	18.18	11	45.21	1.16	0.01	1.12	0.04	1.15	0.02
		ABSciex 5600	11.2	6	29.45	1.28	0.02	1.10	0.23	1.19	0.06
P62258	1433E	ABSciex Qstar Elite	18.12	18	56.86	1.13	0.14	1.12	0.06	1.16	0.02
		ABSciex 5600	28.82	15	52.55	1.13	0.19	1.09	0.31	1.25	0.04
P62277	RS13	ABSciex Qstar Elite	9.34	7	35.10	1.19	0.09	1.22	0.08	1.29	0.10
		ABSciex 5600	9.59	6	22.52	1.34	0.02	1.31	0.02	1.62	0.02
P62701	RS4X	ABSciex Qstar Elite	11.35	7	28.90	1.21	0.04	1.19	0.05	1.25	0.02
		ABSciex 5600	10.26	5	19.01	1.16	0.12	1.12	0.21	1.21	0.06
P62917	RL8	ABSciex Qstar Elite	7.26	4	15.95	1.00	1.00	1.05	0.63	1.23	0.13
		ABSciex 5600	10.2	7	21.79	1.04	0.55	1.11	0.18	1.40	0.00
P83731	RL24	ABSciex Qstar Elite	12.11	7	29.94	1.04	0.52	1.05	0.50	1.08	0.28
		ABSciex 5600	11.11	6	29.94	1.09	0.30	1.13	0.19	1.32	0.02
P84098	RL19	ABSciex Qstar Elite	7.88	4	17.35	1.03	0.71	1.12	0.38	1.17	0.15
		ABSciex 5600	6.51	3	16.84	1.06	0.47	1.23	0.04	1.22	0.05
Q05639	EF1A2	ABSciex Qstar Elite	9.62	23	36.72	1.17	0.10	1.24	0.04	1.20	0.06

		ABSciex 5600	3.97	12	17.49	1.15	0.19	1.16	0.17	1.22	0.10
Q06830	PRDX1	ABSciex Qstar Elite	20.67	14	47.24	1.11	0.15	1.09	0.21	1.12	0.12
		ABSciex 5600	20.09	12	43.22	1.40	0.01	1.36	0.04	1.42	0.02
Q14204	DYHC1	ABSciex Qstar Elite	71.62	37	9.49	1.21	0.00	1.14	0.00	1.19	0.00
		ABSciex 5600	47.5	33	7.96	1.16	0.00	1.09	0.02	1.12	0.00
Q14247	SRC8	ABSciex Qstar Elite	29.45	18	35.45	1.03	0.73	1.04	0.65	1.07	0.18
		ABSciex 5600	35.87	18	28.91	1.03	0.48	1.13	0.09	1.22	0.00
Q15847	APM2	ABSciex Qstar Elite	5.88	4	78.95	1.12	0.22	1.13	0.28	1.14	0.26
		ABSciex 5600	10.19	9	78.95	1.26	0.04	1.26	0.02	1.34	0.05
Q86VP6	CAND1	ABSciex Qstar Elite	17.26	10	8.54	1.09	0.15	1.06	0.36	1.09	0.14
		ABSciex 5600	14.79	9	6.26	1.21	0.04	1.16	0.13	1.20	0.06
Q8IVF2	AHNK2	ABSciex Qstar Elite	8.4	5	7.32	1.06	0.54	1.19	0.06	1.23	0.04
		ABSciex 5600	0.1	2	0.74						
Q9H1E3	NUCKS	ABSciex Qstar Elite	17.42	13	51.85	0.95	0.69	1.16	0.07	1.20	0.06
		ABSciex 5600	20.09	16	52.67	1.05	0.62	1.16	0.06	1.25	0.03
Q9NUQ8	ABCF3	ABSciex Qstar Elite	8	4	8.89	1.01	0.93	1.00	0.97	0.95	0.60
		ABSciex 5600	6.16	3	7.47	1.30	0.03	1.31	0.05	1.22	0.12
Q9UQ80	PA2G4	ABSciex Qstar Elite	22.83	14	31.98	1.13	0.03	1.10	0.04	1.14	0.01
		ABSciex 5600	25.72	13	29.95	1.25	0.01	1.20	0.00	1.22	0.01
Q9Y6B6	SAR1B	ABSciex Qstar Elite	4	2	17.17	1.23	0.36	1.16	0.63	1.17	0.40

		ABSciex 5600	5.25	3	15.15	1.32	0.03	1.16	0.13	1.07	0.43
P15880	RS2	ABSciex Qstar Elite	17.23	8	27.99	1.15	0.01	1.12	0.03	1.16	0.01
		ABSciex 5600	12.83	6	19.80	1.12	0.42	1.22	0.00	1.19	0.01

Table S5 Proteins identified as up and down regulated (>1.20 or <0.83, PVal <0.05) at different time point after IMD0354 treatment using iTRAQ MS/MS

Accession	Symbol	Mass spectrometer	Unused	Peptides95	Cov 95%	30mins	30mins Pval	60mins	60mins Pval	120mins	120mins Pval
O75947	ATP5H	ABSciex Qstar Elite	13.3	7	57.14	0.78	0.00	1.12	0.07	1.05	0.41
		ABSciex 5600	10.58	6	47.20	0.84	0.05	1.27	0.02	1.19	0.04
P02545	LMNA	ABSciex Qstar Elite	64.82	45	49.25	0.73	0.00	1.11	0.00	1.03	0.26
		ABSciex 5600	64.6	38	47.89	0.62	0.00	1.28	0.00	1.09	0.02
P05114	HMG-14	ABSciex Qstar Elite	8.03	6	43.00	0.64	0.02	0.96	0.68	0.94	0.62
		ABSciex 5600	8.12	9	43.00	0.46	0.00	1.27	0.10	1.13	0.25
P07919	QCR6	ABSciex Qstar Elite	5.53	3	39.56	0.67	0.04	1.29	0.09	1.24	0.12
		ABSciex 5600	2.58	2	16.48	0.84	0.28	1.09	0.42	1.15	0.29
P20700	LMNB1	ABSciex Qstar Elite	18.97	10	20.48	0.75	0.03	1.34	0.00	1.21	0.00
		ABSciex 5600	13.43	7	11.09	0.72	0.00	1.43	0.01	1.29	0.01
P62805	H4	ABSciex Qstar Elite	17.49	28	63.11	0.66	0.00	1.01	0.90	0.92	0.43
		ABSciex 5600	13.77	26	51.46	0.51	0.00	1.29	0.11	0.95	0.67
Q71DI3	H32	ABSciex Qstar Elite	13.97	18	47.79	0.81	0.01	1.10	0.12	1.05	0.38
		ABSciex 5600	13.93	11	35.29	0.61		1.68		1.13	

Table S6 Phospho-proteins identified to be up-regulated after IMD0354 treatment using SILAC MS/MS

Assession	Symbol	%Cov (95)	Conf	Sequence	Modifications	Ratio
P27816	MAP4	1.21	99	DMESPTKLDVTLAK	Phospho(S)@4	2.09
Q09666	AHNK	1.61	99	LKSEDGVEGDLGETQSR	Phospho(S)@3	2.09
Q09666	AHNK	1.61	99	SSKASLGSLEGEAEAEASSPK	Phospho(S)@2; Phospho(S)@8	2.07
Q09666	AHNK	1.61	99	SSKASLGSLEGEAEAEASSPK	Phospho(S)@5; Phospho(S)@8	2.00
P46937	YAP1	3.57	99	QASTDAGTAGALTPQHVR	Phospho(S)@3	1.96
Q96TC7	RMD3	3.83	99	SQSLPNSLDYTQTSDPGR	Phospho(S)@3	1.66
P62995	TRA2B	6.94	99	RPHTPTPGIYMGRPTYGSSR	Phospho(T)@4	1.65
Q14157	UBP2L	3.59	99	RYPSSISSSPQKDLTQAK	Phospho(S)@9	1.61
P51858	HDGF	15.00	99	GNAEGSSDEEGKLVIDEPAKEK	Phospho(S)@6; Phospho(S)@7	1.59
Q15019	SEPT2	5.54	99	IYHLPDAESDEDEDFKEQTR	Phospho(S)@9	1.58
P46937	YAP1	3.57	99	QASTDAGTAGALTPQHVR	Gln->pyro-Glu@N-term; Phospho(S)@3	1.49
P46937	YAP1	3.57	99	QASTDAGTAGALTPQHVR	Gln->pyro-Glu@N-term; Phospho(T)@4	1.49
P35222	CTNB1	2.05	99	RTSMGGTQQQFVEGVR	Phospho(T)@7	1.43
P35222	CTNB1	2.05	99	RTSMGGTQQQFVEGVR	Phospho(S)@3	1.43
Q9NYF8	BCLF1	4.13	99	LKDLFDYSPPLHK	Phospho(S)@8	1.41
Q92538	GBF1	0.91	99	GYTSDSEVYTDHGRPGK	Phospho(S)@4	1.37
Q9H1E3	NUCKS	8.23	99	TPSPKEEDEEPESPPEKK	Phospho(S)@13	1.28
Q9UHD8	SEPT9	2.73	99	SFEVEEVETPNSTPPR	Phospho(S)@1	1.28
Q8IYB3	SRRM1	3.65	99	KETESEAEDNLDDLEK	Phospho(S)@5	1.27
P25788	PSA3	5.49	99	ESLKEEDESDDDNM	Phospho(S)@9	1.21
Q15149	PLEC	0.38	99	SSSVGSSSSYPISPAVSR	Phospho(S)@3	1.21
P42167	LAP2B	14.10	99	QNGSNDSDRYSDNEEDSKIELK	Gln->pyro-Glu@N-term; Phospho(S)@11	1.21
Q13765	NACA	16.28	99	VQGEAVSNIQENTQTPTVQEESEEEEVDETGVEVK	Phospho(T)@17	1.21

Table S7 Phospho-proteins identified to be down-regulated after IMD0354 treatment using SILAC MS/MS

Assession	Symbol	%Cov (95)	Conf	Sequence	Modifications	Ratio
P27824	CALX	11.82	97.7	AEEDEILNRSPR	Phospho(S)@10	0.83
Q9NYF8	BCLF1	4.13	99	RIDISPSTLR	Phospho(S)@7	0.82
O94826	TOM70	3.95	99	ASPAPGSGHPEGPGAHLDMNSLDR	Phospho(S)@2;Oxidation(M)@19	0.78
Q13547	HDAC1	6.85	99	MLPHAPGVQMQAIPEDAIPEESGDEDEDDPDKR	Phospho(S)@22	0.78
P35659	DEK	5.60	99	EESEEEEDEDDEEEEEEEKEK	Phospho(S)@3	0.77
O94826	TOM70	3.95	99	ASPAPGSGHPEGPGAHLDMNSLDR	Phospho(S)@2	0.74
Q14157	UBP2L	3.59	99	SPAVATSTAAPPPPSSPLPSK	Phospho(S)@16	0.74
P49736	MCM2	1.77	99	GLLYDSDEEDEERPAR	Phospho(S)@6	0.73
Q92733	PRCC	4.07	99	IAAPELHKGDSDSEEDEPTK	Phospho(S)@11;Phospho(S)@13	0.72
O15173	PGRC2	10.76	99	LLKPGEEPSEYTDEEDTKDHNKQD	Phospho(T)@12	0.72
P13861	KAP2	5.69	99	VADAKGDSESEEDEDLEVPVPSR	Phospho(S)@8; Phospho(S)@10	0.70
Q14980	NUMA1	0.90	99	TQPDGTSVPGEPASPISQR	Phospho(S)@17	0.69
P42167	LAP2B	14.10	99	SSTPLPTISSSAENTR	Phospho(T)@3	0.68
Q09666	AHNK	1.61	99	LPSGSGAASPTGSAVDIR	Phospho(S)@9	0.66
P42167	LAP2B	14.10	99	GPPDFSSDEEREPTPVLGSGAAAAGR	Phospho(S)@6; Phospho(S)@7	0.62
Q13247	SFRS6	3.20	99	SNSPLPVPPSK	Phospho(S)@3	0.60
Q9H3N1	TMX1	7.50	99	KVEEEQEADEEDVSEEEAESK	Phospho(S)@14	0.60
Q9NYF8	BCLF1	4.13	99	YSPSQNSPIHHIPSR	Phospho(S)@2; Phospho(H)@10	0.59
Q9P206	K1522	1.55	99	ASPVPAPSSGLHAAVR	Phospho(S)@2	0.57
Q09666	AHNK	1.61	99	GHYEVTGSDDETGKLQGSGVSLASK	Phospho(S)@8	0.45

Table S8 siRNA Screen

Gene Symbol	Description	siRNA target sequence
HNRNPC	heterogeneous nuclear ribonucleoprotein C (C1/C2)	AACGTCAGCGTGTATCAGGAA
RALY	RNA binding protein, autoantigenic (hnRNP-associated with lethal yellow homolog)	AACGTACCTGTCAAGCTCTTT
ACIN1	apoptotic chromatin condensation inducer 1	CTCGCTGCCCAAATCATTCAA
HNRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1	TTGGATTATTTAATAACATTA
SAFB	scaffold attachment factor B	ACGGACTGTAGTAATGGATAA
C1QBP	complement component 1, q subcomponent binding protein	CACGGTCACTTTCAACATTAA
CPD	carboxypeptidase D	CAGGACTGGAACTATTTACAA
CHTOP	chromatin target of PRMT1	GAGGACGAGGCCTACCCATAA
RBM10	RNA binding motif protein 10	CTCCAACGTGCGCGTCATAAA
HMGN1	high mobility group nucleosome binding domain 1	GAAGCCAAGTCTGATTAATAA
HNRNPL	heterogeneous nuclear ribonucleoprotein L	AAAGCCTACACGCTTGAATGT
HNRNPUL2	heterogeneous nuclear ribonucleoprotein U-like 2	ACGGGCGAGATTATGAGTACA
PBXIP1	pre-B-cell leukemia homeobox interacting protein 1	CTGGGAGATGTAGCAAATTGA
HNRNPH3	heterogeneous nuclear ribonucleoprotein H3 (2H9)	AGCGACCGGGACCATATGATA
ATP5H	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d	ATGACTTTGAGAAGAAGTTTA
ZC3H14	zinc finger CCCH-type containing 14	AAGACCGGACTGCACATTCTA
ATP5A1	ATP synthase alpha chain, mitochondrial	TTGGCTGGATTTGAAGCTTAA
PNN	pinin, desmosome associated protein	CCGGAGGATGATGATGTTAAA
SNW1	SNW domain containing 1	CCCGATGAAGAAGCTATTAAA
SON	SON DNA binding protein	CCGGTCTATGATGTCGTCATA
CCAR1	cell division cycle and apoptosis regulator 1	ATGGCTAGCCCTAGTATGGAA
SF3B2	splicing factor 3b, subunit 2, 145kDa	CCCATCGTATCCCAACCTGAA
ALYREF	Aly/REF export factor	TTGCTGAATTTGGAACGCTGA
SF3A1	splicing factor 3a, subunit 1, 120kDa	CAGGATAAGACGGAATGGAAA
RBM25	RNA binding motif protein 25	ATCGCAGTCGATCAAGAGAAA
NOP58	NOP58 ribonucleoprotein homolog (yeast)	AACCATGTACCAGCACAGCAA
NOP56	NOP56 ribonucleoprotein homolog (yeast)	CCCAGTTTATTGGAAACCGAA
HNRNPA3	heterogeneous nuclear ribonucleoprotein A3	AAGGGCAATAAGCATTAGGTA
SATB1	SATB homeobox 1	TCCAAGCAATTCATACTATAA
ILF3	interleukin enhancer binding factor 3, 90kDa	CAAGCTGCACGTGGCCGTTAA

CKAP4	cytoskeleton-associated protein 4	AGGGCGCGGATTTAAAGTCCA
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	AAGGTTGTCGATCTGCTAGCT
MYL6	myosin, light chain 6, alkali, smooth muscle and non-muscle	ACCTATGAGGATTATGTCGAA
RBMX	RNA binding motif protein, X-linked	CTCAATGTTCTCAGTACGAAA
PSMB3	proteasome (prosome, macropain) subunit, beta type, 3	AACGGTTTGGCCCTTACTACA
ATP5J	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F6	CACGGATTAGTTGTACAACTA
TPR	translocated promoter region (to activated MET oncogene)	CAGGAGGTTTCTAGAGAACAA
SMARCC2	SWI/SNF complex subunit SMARCC2	CAGGTGGATGCTGAGAGTCGA
NUMA1	nuclear mitotic apparatus protein 1	CAGCAGCGCATTGACCGCCTA
SF3A3	splicing factor 3a, subunit 3, 60kDa	CAGCGACATCTCACTCATGAA
UBTF	upstream binding transcription factor, RNA polymerase I	CAGGACCGTGCAGCATATAAA
SFPQ	splicing factor proline/glutamine-rich	AATATGGAGAACCAGGAGAAG
EFHD1	EF-hand domain family, member D1	CTGGGTAATGGTGGGTCTTAA
LMNB1	lamin B1	AACGCGCTTGGTAGAGGTGGA
CHERP	calcium homeostasis endoplasmic reticulum protein	CTGGATGTTCAGCAATGCCAA
CANX	calnexin	CCTGCATAATGTTAAGCTTTA
TP53BP1	tumor protein p53 binding protein 1	TTGTTTGACCAGAGCAATAAA
NDUFB11	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11	CAGCAAGATCCAGCTGCCAGA
FBL	fibrillarin	ATCGTTGGTCCGGATGGTCTA
LMNA	lamin A/C	CCAGGAGCTTCTGGACATCAA
SARNP	SAP domain containing ribonucleoprotein	ACGGTGGAGCTCCATAAGCTA
SRSF7	serine/arginine-rich splicing factor 7	CTGGATGATCTTTATACTAAT
PRPF19	PRP19/PSO4 pre-mRNA processing factor 19 homolog	AAGTACATTGCGGAGAATGGT
SRSF9	serine/arginine-rich splicing factor 9	AATGGTTATGATTATGGCCAG
SRSF3	serine/arginine-rich splicing factor 3	CTCGTAGTCGATCTAGGTCAA
SRSF1	serine/arginine-rich splicing factor 1	TTGGCAGGATTTAAAGGATCA
RPS4X	ribosomal protein S4, X-linked	CTGGAGGTGCTAACCTAGGAA
ANXA5	annexin A5	CGCGAGACTTCTGGCAATTTA
PSMC5	proteasome (prosome, macropain) 26S subunit, ATPase, 5	AAGGTACATCCTGAAGGTAAA
RPS9	ribosomal protein S9	ACGGCGTCTGTTCGAAGGCAA
RPS3	ribosomal protein S3	CGGCAAGATGGCAGTGCAAAT
GSTM3	glutathione S-transferase mu 3 (brain)	AACGGAAGAAGTTACCCTTAA

AHNAK2	AHNAK nucleoprotein 2	CCCGAGGTAGCCGGCCTCAAA
BLVRB	biliverdin reductase B (flavin reductase (NADPH))	CAAGAGCTTCAAATTACTCTA
CSE1L	CSE1 chromosome segregation 1-like (yeast)	CAGGATAATGTTATCAAAGTA
DYNC1H1	dynein, cytoplasmic 1, heavy chain 1	CAGGTGGGTGTACATTACGAA
DEK	DEK oncogene	CAGAATATAATGTGCAGGCAA
MCM2	minichromosome maintenance complex component 2	CTCATTGGAGATGGCATGGAA
BCLAF1	BCL2-associated transcription factor 1	CACGCTTTACGATATTATAAA
TOMM70A	translocase of outer mitochondrial membrane 70 homolog A	AAGACAATAAGAAGGAATGTT
TMPO	thymopoietin	AAAGATGATCTAGATGTAACA
HDGF	hepatoma-derived growth factor	AAGAGCTGTGTGGAAGAGCCT
PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3	AATGACCTGCCGTGATATCGT
YAP1	Yes-associated protein 1	CAGGTGATACTATCAACCAAA
NACA	nascent polypeptide-associated complex alpha subunit	AAGAAGGCACGGAAGGCTATG